Detoxification of 3,4-dichloroaniline in soybean by n-malonylation

Lao, Si-houy

DETOXIFICATION OF 3,4-DICHLOROANILINE IN SOYBEAN BY *N*-MALONYLATION

Si-houy LAO

Ph.D. Thesis (2003)

UNIVERSITY OF DURHAM

I confirm that no part of the material offered has previously been submitted by me for a degree in this or in any other University. If material has been generated through joint work, my independent contribution has been clearly indicated. In all other cases, material from the work of others has been acknowledged and quotations and paraphrases suitably indicated.

Si-houy Lao

October 2003

ACKNOWLEDGMENTS

I would like to thank my supervisors, in order of appearance: Julian, Freddie and Robert. I remember particularly my long discussions with Julian... about exotic food, my many corrections and tears crises with Freddie and the good time I had in Robert's laboratory, despite all the hard work. Each, in their own way, has taught me a lot. I have definitely come out of this PhD stronger than I was! A very special thank you to Freddie for all her support during the writing-up of this thesis and for cheering me up every time I needed it. I really appreciated.

Many thanks to all the people who made my everyday life pleasant during those years of hardship. Special thanks to John Andralojc who has always gone out of the way to help. Thank you to Tine and Cristina for all the fun we had and to Xiao-Li for being so lovely and nice; I am very happy to have met you all.

Thank you to my family who never asked anything and always trusted me. I would like to thank particularly my mother for having made of me the person I am today and for loving me enough to have let me come and study in England.

Pour Pierre,

Merci de m'avoir écoutée me plaindre pendant tant d'heures et de toujours avoir été là quand j'avais besoin de toi, malgré la distance. Sans toi, cette thèse n'existerait pas.

ABBREVIATIONS

ABC	ATP-binding cassette
ACC	aminocyclopropane-1-carboxylic acid
ACCase	acetyl-CoA carboxylase
ACC-*N*-MT	1-aminocyclopropane-1-carboxylate *N*-malonyltransferases
actD	actinomycin D
ATP	adenosine triphosphate
ATPase	ATP synthase
BSA	bovine serum albumin
Bq	Becquerel
cDNA	complementary DNA
Ci	Curie
CH	cycloheximide
DCA	3,4-dichloroaniline
d	day
DEAE	diethyl-amino-ethyl
DIDS	4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid
DiGE	differential gel electrophoresis
DMSO	dimetylsulphoxide
dpm	disintegration per minute
DTT	dithiothreitol
G-DCA	*N*-β-D-glucopyranosyl-3,4-dichloroaniline
GSH	glutathione
GST	glutathione S-transferases
GT	glucosyltransferase
HCl	hydrochloric acid
hGSH	homoglutathione
HPLC	High Performance Liquid Chromatography
IVEC	*in vitro* expression cloning
Kat	Katal

K_m	Michaelis-Menten constant
K_{ow}	1-octanol/water partition coefficient
LSC	liquid scintillation counting
MACC	malonyl-aminocyclopropane-1-carboxylic acid
M-CoA	malonyl-CoA
M-DCA	*N*-malonyl-3,4-dichloroaniline
MDR	multi-drug resistance protein
M-G-DCA	6''-O-malonyl-DCA-*N*-(β-D-glucopyranosyl)-3,4-dichloroaniline
MRP	multidrug resistance associated protein
MS	Murashige and Skoog basal medium
MT	malonyltransferase
NBF	nucleotide binding fold
N-GT	*N*-glucosyltransferase
N-MT	*N*-malonytransferase
O-GT	*O*-glucosytransferase
O-MT	*O*-malonytransferase
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
PVPP	polyvinylpolypyrrolidone
Rf	migration coefficient
RRL	rabbit reticulocyte lysate
SA	specific activity
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SH	Schenk and Hildebrandt basal salt mixture
TEMED	*NNN'N'*-tetramethylethylenediamine
THC	tetrahydroxychalcone
TLC	thin layer chromatography
TMS	transmembrane spanning
UDPG	uridine diphosphate glucose

DETOXIFICATION OF 3,4-DICHLOROANILINE IN SOYBEAN BY *N*-MALONYLATION

Si-houy LAO, Ph.D. (2003)

ABSTRACT

3,4-Dichloroaniline (DCA), the degradation product of certain herbicides, is not readily degraded by micro-organisms and, due to its persistence in the environment, is considered to be a reference xenobiotic. Here, the metabolic fate of [UL-^{14}C]-3,4-DCA was investigated in soybean (*Glycine max* var. Chapman) plants over a 48 h period following treatment via the root media. DCA was rapidly taken up and metabolised to *N*-malonyl-DCA. Synthesis occurred in the roots and the conjugate was largely exported into the culture medium, a smaller proportion being retained within the plant tissue. Once exported, the DCA metabolites present in the medium were not readily taken up by soybean roots. Conjugation and export of DCA therefore constitute an effective detoxification mechanism for the plant.

A radiometric assay for DCA-*N*-malonyltransferase (E.C.2.3.1.114; DCA-*N*-MT) was developed and used to follow DCA-*N*-MT activity through a four-step protocol, in which DCA-*N*-MT was purified 400-fold from soybean roots. SDS-PAGE analysis and gel filtration chromatography suggested that DCA-*N*-MT is a 52 ± 2 kDa protein. Following treatments with 100 µM DCA for 24 h, DCA-*N*-MT activity in soybean roots increased from 44.6 ± 8.1 nkat.g^{-1} to 104 ± 4.9 nkat.g^{-1} but did not vary significantly in suspension-cultured cells (332.9 ± 38.9 nkat.g^{-1}). Kinetic studies suggested that this increase in activity could be due to *de novo* protein synthesis. Partially-purified DCA-*N*-MT was therefore subjected to differential gel electrophoresis (DiGE) analysis to identify proteins which increased in abundance in response to DCA pre-treatment. Although a clear candidate for DCA-*N*-MT was not detected, five differentially expressed proteins were identified by mass spectrometry.

LIST OF FIGURES

Figure 1.1: Detoxification of xenobiotics in plants 3

Figure 1.2: Enzymes involved in the metabolism of 3,4-dichloroaniline in plants 19

Figure 2.1: Growth curve for suspension-cultured soybean cells 27

Figure 2.2: Ethyl acetate quench correction for liquid scintillation analyser 31

Figure 2.3: Strategies employed for the purification of DCA-*N*-MT from soybean roots 38

Figure 3.1: Localisation of radiolabelled compounds in soybean plants 50

Figure 3.2: Distribution of [UL-^{14}C]-DCA metabolites between the growth medium and the soybean seedling 51

Figure 3.3: Identification of [UL-^{14}C]-DCA metabolites by TLC 53

Figure 3.4: Time-course of [UL-^{14}C]-DCA uptake and distribution in soybean 55

Figure 3.5: Time-course analysis of [UL-^{14}C]-DCA metabolites in the growth media 57

Figure 3.6: Time-course analysis of [UL-^{14}C]-DCA metabolites in soybean roots 60

Figure 3.7: Time-course of [UL-^{14}C]-DCA uptake and distribution of label in *Arabidopsis thaliana* 62

Figure 3.8: Analysis of [UL-^{14}C]-DCA metabolites in *Arabidopsis thaliana* 63

Figure 3.9: Partition of radiolabelled compounds between *Arabidopsis*, soybean and nutrient medium, when grown with *Arabidopsis* and soybean DCA-metabolites 68

Figure 3.10: Effect of various inhibitors on M-DCA efflux from soybean roots 70

Figure 3.11: Accumulation of weak acids within cells, by ion trapping 75

Figure 4.1: Principle of 3,4-dichloroaniline-*N*-malonyltransferase assay 80

Figure 4.2: Assessment of two solvents for the separation of DCA-*N*-MT reaction products by TLC 83

Figure 4.3: Selection of radiolabelled substrate for DCA-*N*-MT assay 84

Figure 4.4: Effects of temperature, pH and buffer on DCA-*N*-MT activity in soybean roots 86

Figure 4.5: Effect of 0.1 % (w/v) bovine serum albumin on DCA-*N*-MT

activity in soybean roots 88
Figure 4.6: Linearity of DCA-*N*-MT assay, with respect to protein concentration and time 90
Figure 4.7: Linearity of DCA-*N*-MT assay with cultured cell extract, with respect to protein concentration and time 91
Figure 4.8: Recovery of DCA-*N*-MT during ammonium sulphate precipitation 95
Figure 4.9: Elution profile of DCA-*N*-MT from a DEAE-Sepharose column 97
Figure 4.10: Elution profile of DCA-*N*-MT from a phenyl-Sepharose column 99
Figure 4.11: Elution of DCA-*N*-MT from dye-ligand columns 102
Figure 4.12: Elution profile of DCA-*N*-MT from a Cibacron Blue F3G-A column 104
Figure 4.13: SDS-PAGE of DCA-*N*-MT during purification procedure 1 107
Figure 4.14: Elution profile of DCA-*N*-MT from a DEAE-Sepharose column, using a linear NaCl gradient 110
Figure 4.15: Elution profile of DCA-*N*-MT from a Resource Q column 112
Figure 4.16: Elution profile of DCA-*N*-MT from a Sephacryl S-100 column 113
Figure 4.17: SDS-PAGE of DCA-*N*-MT during purification procedure 2 115
Figure 4.18: SDS-PAGE of DCA-*N*-MT during purification procedure 2.2 117
Figure 5.1: Time-course study of DCA-*N*-MT activity in suspension-cultured soybean cells, following DCA treatments 125
Figure 5.2: Time-course study of DCA-*N*-MT activity in soybean roots, following DCA treatments 126
Figure 5.3: Kinetic studies of DCA-*N*-MT activity in soybean tissues 128
Figure 5.4: DCA-*N*-MT activity in *in vitro* translation samples 132
Figure 5.5: Elution profile of DCA-*N*-MT from control and DCA-treated soybean roots, after a DEAE-Sepharose column from control and DCA-treated soybean roots 134
Figure 5.6: Elution profile of DCA-*N*-MT from control and DCA-treated soybean roots, after a Sephacryl S-100 column from control and DCA-treated soybean roots 135
Figure 5.7: SDS-PAGE of DCA-*N*-MT with and without DCA-treatment 138
Figure 5.8: DiGE analysis of partially purified DCA-*N*-MT 140
Figure 5.9: Hypothetical scheme showing the effect of DCA pre-treatment on enzymes involved in the synthesis of UDP-glucose 145

LIST OF TABLES

Table 1.1: New classification of plant glutathione *S*-transferases 8

Table 1.2: Properties of purified *N*-malonyltransferases 21

Table 3.1: Variation of DCA metabolites with time, in soybean 58

Table 3.2: Variation of DCA metabolites with time, in *Arabidopsis thaliana* 64

Table 3.3: DCA-*N*-MT and DCA-*N*-GT activities in soybean and Arabidopsis tissues 66

Table 4.1: Developmental profile of DCA-*N*-MT activity in soybean roots 93

Table 4.2: Comparison of dye-ligand chromatography columns for DCA-*N*-MT purification 101

Table 4.3: Balance sheet for the purification of DCA-*N*-MT following procedure 1 106

Table 4.4: Balance sheet for the purification of DCA-*N*-MT following procedure 2 109

Table 4.5: Balance sheet for the purification of DCA-*N*-MT following procedure 2.2 116

Table 5.1: Balance sheet for the purification of DCA-*N*-MT from control and DCA-treated soybean roots 136

Table 5.2: Summary of DiGE analysis of soybean proteins whose abundance is regulated by DCA treatment 141

CHAPTER 1 1

INTRODUCTION 1

1.1. XENOBIOTIC DETOXIFICATION 2

1.1.1. UPTAKE OF XENOBIOTICS BY PLANTS 2

1.1.2. DETOXIFICATION: PHASE I 4

1.1.3. DETOXIFICATION: PHASE II 5

1.1.3.1. Glutathione S-transferases 5

1.1.3.2. Glucosyltransferases 7

1.1.3.3. Malonyltransferases 11

1.1.4. DETOXIFICATION: PHASE III 13

1.1.4.1. Sequestration in the vacuole 13

1.1.4.2. Sequestration in the apoplast 15

1.1.5. DETOXIFICATION: PHASE IV 16

1.2. 3,4-DICHLOROANILINE, A MODEL XENOBIOTIC 17

1.3. *N*-MALONYLTRANSFERASES, CURRENT KNOWLEDGE 18

1.4. AIMS 23

CHAPTER 2 24

MATERIALS AND METHODS 24

2.1. CHEMICALS 24

2.1.1. TISSUE CULTURE AND BIOCHEMICAL STUDIES 24

2.1.2. RADIOCHEMICALS 24

2.1.3. SYNTHESIS OF 3,4-DICHLOROANILINE CONJUGATES 25

2.2. PLANT MATERIAL 25

2.2.1. SOYBEAN TISSUE CULTURE 25

2.2.2. SOYBEAN 26

2.2.3. ARABIDOPSIS 28

2.3. DCA METABOLISM STUDIES 28

2.3.1. TREATMENT OF SOYBEAN PLANTS WITH DCA 28

2.3.2. EXTRACTION OF RADIOLABELLED DCA METABOLITES FROM PLANT MATERIAL 28

2.3.3. EXTRACTION OF DCA METABOLITES FROM GROWTH MEDIA 29

2.3.4. LIQUID SCINTILLATION COUNTING (LSC) 30

2.3.4.1. Quench curve 30

2.3.4.2. Counting of samples 30

2.3.5. THIN LAYER CHROMATOGRAPHY (TLC) 32
2.3.6. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) 32
2.3.7. VISUALISATION OF DCA METABOLITES BY PHOSPHOR-IMAGING 33
2.3.8. DCA-*N*-MT ASSAY 33
2.3.8.1. Initial DCA-N-MT assay 33
2.3.8.2. Optimised DCA-N-MT assay 33
2.3.8.3. Final DCA-N-MT assay 34
2.3.9. *N*-GLUCOSYLTRANSFERASE ASSAY 34
2.3.10. ASSAY FOR THE PRESENCE OF 6''-*O*-MALONYL-DCA-*N*-(β-D-GLUCOPYRANOSYL)-3,4-DICHLOROANILINE (M-G-DCA) 34
2.3.11. TIME-COURSE OF DCA UPTAKE 35
2.3.12. CROSS-FEEDING EXPERIMENT 35
2.3.13. EFFECT OF INHIBITORS ON M-DCA EFFLUX FROM SOYBEAN ROOTS 36
2.4. PURIFICATION OF 3,4-DICHLOROANILINE-DCA-*N*-MALONYLTRANSFERASE 36
2.4.1. PREPARATION OF EXTRACTS 36
2.4.2. PURIFICATION PROCEDURE 1 37
2.4.3. PURIFICATION PROCEDURE 2 39
2.4.4. DETERMINATION OF PROTEIN CONCENTRATION 40
2.4.4.1. Using a microtitre plate 40
2.4.4.2. Using 1 ml cuvettes 41
2.4.5. SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) 41
2.5. FURTHER CHARACTERISATION OF DCA-*N*-MT 42
2.5.1. EFFECT OF DCA ON DCA-*N*-MT ACTIVITY 42
2.5.2. KINETIC STUDIES 43
2.5.3. EFFECTS OF INHIBITORS ON DCA-*N*-MT ACTIVITY 43
2.5.4. *IN VITRO* TRANSLATION OF SOYBEAN RNA 44
2.5.4.1. RNA extraction and quantification 44
2.5.4.2. In vitro translation 45
2.6. FLUORESCENCE TWO-DIMENSIONAL DIFFERENTIAL GEL ELECTROPHORESIS (2-D DIGE) 45
2.6.1 SAMPLE PREPARATION 45
2.6.2 DIGE LABELLING 46
2.6.3 ELECTROPHORETIC ANALYSIS 46

CHAPTER 3 48

METABOLISM OF 3,4-DICHLOROANILINE IN SOYBEAN 48

3.1. INTRODUCTION 48

3.2. RESULTS 49

3.2.1. DISTRIBUTION OF RADIOLABEL FOLLOWING [UL-^{14}C]-DCA TREATMENT 49

3.2.2. IDENTIFICATION OF [UL-^{14}C]-DCA METABOLITES IN SOYBEAN 52

3.2.3. TIME-COURSE STUDY OF DCA METABOLISM IN SOYBEAN PLANTS 54

3.2.3.1. Partition of radiolabelled DCA-metabolites 54

3.2.3.2. Variation of radiolabelled DCA-metabolites with time 56

3.2.4. COMPARISON WITH DCA METABOLISM IN *ARABIDOPSIS* ROOT CULTURES 59

3.2.5. TRANSFERASE ACTIVITIES 65

3.2.6. TRANSPORT STUDIES 65

3.2.6.1. Cross-feeding 65

3.2.6.2. Effect of transport inhibitors on the efflux of M-DCA 69

3.3. DISCUSSION 71

3.4. SUMMARY 77

CHAPTER 4 78

PURIFICATION OF DCA-*N*-MT FROM SOYBEAN ROOTS 78

4.1. INTRODUCTION 78

4.2. RESULTS 78

4.2.1. DEVELOPMENT AND OPTIMISATION OF DCA-*N*-MT ASSAY 78

4.2.1.1. Basis for DCA-N-MT assay 79

4.2.1.2. Qualitative analysis of the reaction products by TLC 81

4.2.1.2.1. Solvent system 1 82

4.2.1.2.2. Solvent system 2 82

4.2.1.2.3. Choice of radiolabel 82

4.2.1.3. Quantitative analysis of the reaction products by LSC 85

4.2.1.4. Optimisation of DCA-N-MT assay 85

4.2.1.4.1. Effect of temperature 85

4.2.1.4.2. Effect of pH and buffer 87

4.2.1.4.3. Effect of bovine serum albumin (BSA) 87

4.2.1.4.4. Linearity of the assay 89

4.2.2. PURIFICATION PROCEDURE 89

4.2.2.1. Choice of starting material 92
4.2.2.2. Purification procedure 1 94
4.2.2.2.1. Crude extracts 94
4.2.2.2.2. Centricon filters 96
4.2.2.2.3. Ion exchange chromatography 96
4.2.2.2.4. Hydrophobic interaction chromatography 98
4.2.2.2.5. Dye-ligand chromatography 98
4.2.2.2.6. Assessment of purification procedure 1 105
4.2.2.3. Purification procedure 2 108
4.2.2.3.1. Protamine sulphate precipitation 108
4.2.2.3.2. Additional steps 111
4.2.2.3.3. Assessment of purification procedure 2 114
4.3. DISCUSSION **118**
4.4. SUMMARY **119**

CHAPTER 5 **121**

FURTHER CHARACTERISATION OF DCA-*N*-MT: TOWARDS A MOLECULAR IDENTIFICATION **121**
5.1. INTRODUCTION **121**
5.2. EFFECT OF DCA ON DCA-*N*-MT ACTIVITY **123**
5.2.1. SUSPENSION-CULTURED CELLS 123
5.2.2. PLANTS 124
5.3. KINETIC STUDIES **124**
5.4. MECHANISM OF INDUCTION OF DCA-*N*-MT ACTIVITY **129**
5.4.1. EFFECTS OF INHIBITORS 129
5.4.2. *IN VITRO* TRANSLATION 130
5.5. IDENTIFICATION OF PROTEINS INDUCED BY DCA PRE-TREATMENT **131**
5.5.1. PURIFICATION OF DCA-*N*-MT FROM CONTROL AND DCA-TREATED SOYBEAN ROOTS 133
5.5.2. DIFFERENTIAL GEL ELECTROPHORESIS (DIGE) 137
5.6. DISCUSSION **142**
5.7. SUMMARY **146**

CHAPTER 6 148

DISCUSSION 148

6.1. THE METABOLIC FATE OF DCA IN SOYBEAN 148

6.2. EXPORT OF M-DCA AND ITS IMPLICATIONS FOR THE ENVIRONMENT 149

6.3. TOWARDS THE ISOLATION OF DCA-*N*-MT 151

6.4. IMPORTANCE OF THE CO-SUBSTRATES 153

6.5. XENOBIOTIC DETOXIFICATION AND SECONDARY METABOLISM, WHERE DOES THE BOUNDARY LIE? 154

6.6. FUTURE WORK 155

REFERENCES 157

APPENDICES 175

Chapter 1

INTRODUCTION

Weed control by herbicides is an integral part of modern commercial agriculture and because of cost-effectiveness, it is likely to remain an important agronomic strategy for the foreseeable future. The most effective weed-control measures employ selective herbicides that can suppress or kill yield-reducing weeds without harming the crop. One of the most common factors responsible for herbicide selectivity is the ability of a crop to detoxify (metabolise) the herbicide to non-phytotoxic substances, faster than the competing weed (Barrett *et al.*, 1997). However, the continuous use of herbicides over several years can lead to the selection and enrichment of genes that confer resistance in weed populations. Enhanced metabolism of herbicides by some herbicide-tolerant weed populations is a common resistance mechanism and herbicide resistance is an important agricultural and environmental problem.

Plants are continuously exposed to potentially toxic compounds. These xenobiotics consist of synthetic, man-made compounds such as herbicides and pesticides, or can be natural products synthesised by plants themselves (secondary metabolites). Many of these compounds are important commercially as pharmaceuticals, dyes, flavourings or natural plant protection agents. Since the metabolic pathways used by plants to deal with these endogenous natural products overlap with, or are parallel to the pathways they employ for the detoxification metabolism of herbicides, a better characterisation of the mechanisms of herbicide metabolism would contribute to understanding the selectivity of herbicides, the evolution of herbicide resistance and the synthesis of natural products.

1.1. Xenobiotic detoxification

Many xenobiotics are lipophilic and readily accumulate within plant tissues up to toxic levels. To survive, some plants possess enzymes that catalyse chemical transformations of xenobiotics to non-toxic metabolites, a process known as detoxification (Kreuz *et al.*, 1996; Coleman *et al.*, 1997; Cole and Edwards, 2000). As illustrated by figure 1.1, these transformations can be grouped into three or more sequential phases, which are described in more detail below.

1.1.1. Uptake of xenobiotics by plants

In order to enter plants, toxic compounds must overcome the natural protective barriers of plants. The uptake and transport of chemicals by plants has been widely studied, using a range of xenobiotics and plant species. In soybean seeds (Rieder *et al.*, 1969) and soybean roots (Moody *et al.*, 1970), the uptake of herbicides was thought to be due to as yet undefined physical processes. Subsequently, the importance of physico-chemical parameters such as lipophilicity/water solubility and acid strength was demonstrated and a model for the uptake of chemicals by roots was developed (Briggs *et al.*, 1982; Bromilow and Chamberlain, 1995; Chamberlain *et al.*, 1996). These studies showed that in general, the most important property controlling the movement of a chemical in plants is its lipophilicity, which is usually assessed using the octan-1-ol/water partition coefficient, K_{ow}. The pKa of a chemical is also an important criterion, as it is more difficult for an ionised molecule to partition through biological membranes. This is manifested in a pH-dependent uptake and transport behaviour of charged compounds (Sterling, 1994; Bromilow and Chamberlain, 1995). There are essentially two routes of solute uptake into plant roots: symplastic and apoplastic. In general, a chemical is believed to enter roots via the apoplast in the cortex. When it reaches the endodermis, where the apoplastic route is hindered due to the presence of the Casparian strip, the chemical has to enter the cell through the plasma membrane. This is where its log K_{ow} plays an important role: a highly lipophilic compound would partition easily through the membrane but its transport in the xylem would be difficult. Conversely, a chemical with a low log K_{ow} would be

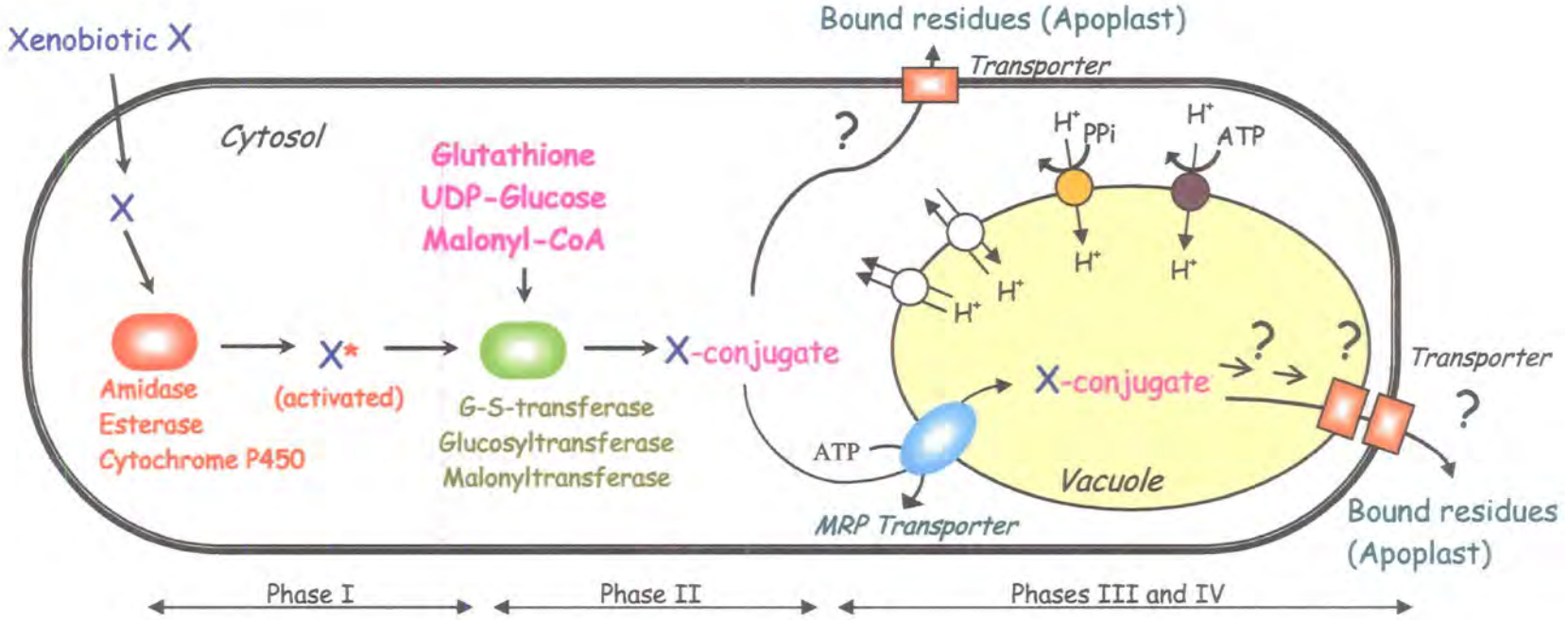


Figure 1.1: Detoxification of xenobiotics in plants.

In phase I (activation), the xenobiotic X is activated via non-synthetic processes such as oxidations, reductions and hydrolyses and prepared for phase II reactions. In phase II (conjugation), the activated compound X* is conjugated to hydrophilic molecules (e.g. glucose, glutathione or malonate), thus hampering its movement within the plant and generally rendering it less harmful. In phase III (compartmentation), X-conjugates are transported into the vacuole by ABC-transporters and H^+-coupled transporters, or into the apoplast (bound residues) via plasma membrane transporters. In phase IV, previously sequestered conjugates are metabolised further. **?** indicates that the identity of enzyme or transporter is unknown. Proton pumps: H^+-pyrophosphatase (yellow circle) and H^+-ATPase (purple circle). Secondary transporters present in the tonoplast: proton-dependent symport and antiport (white circles).

rapidly transported in the xylem but its uptake by the root would be slower (assuming the absence of transporters).

The uptake of chemicals following foliar application is generally slower than that in roots, as aerial plant parts are covered by a waxy cuticle, which serves as a barrier against water losses and pathogen invasions. However, since the xenobiotic 3,4-dichloroaniline generally enters plants via their roots, its fate in leaves will not be dealt with here. Information about the uptake and transport of herbicides applied to leaves, such as the phenoxyacetic acids, is nevertheless available elsewhere (Pilmoor and Gaunt, 1981).

1.1.2. Detoxification: phase I

The first phase of xenobiotic detoxification can be considered as a preparation phase, during which the xenobiotic is chemically activated to enable its conjugation to an endogenous hydrophilic molecule in the subsequent detoxification phase. Phase I reactions usually involve hydrolysis or oxidation. Hydrolysis is catalysed by amidases or esterases (Incledon and Hall, 1997) but most reactions are oxidations, catalysed by the haem proteins, cytochrome P450 monooxygenases (Kreuz *et al.*, 1996; Aoki *et al.*, 2000), which incorporate an oxygen atom from molecular oxygen into a substrate (R-H), the second oxygen atom being converted to water. This reaction is NADPH-dependent (equation 1.1):

$$R\text{-}H + O_2 + NADPH + H^+ \rightarrow R\text{-}OH + H_2O + NADP^+ \qquad \text{(Equation 1.1)}$$

Cytochrome P450s exist as many isoforms with molecular masses ranging from 45 to 62 kDa. They form one of the largest classes of plant enzymes and several hundred are found in any plant species. This multiplicity is reflected in their wide range of functions, not only in the secondary metabolism where they are involved in various biosynthetic pathways, such as those for fatty acids, flavonoids or alkaloids (Bolwell *et al.*, 1994; Schuler, 1996; Guengerich, 2003), but also in xenobiotic detoxification. To

date, over 900 plant cytochromes P450 have been listed in the Cytochrome P450 Database (http://drnelson.utmem.edu/ cytochromep450.html).

The first direct evidence of cytochrome P450 involvement in herbicide detoxification dates back to the end of the 1960s. Subsequently, bacterial and mammalian P450 genes have been used to engineer herbicide resistance in plants (Werck-Reichhart *et al.*, 2000). Recently, the expression of a soybean cytochrome P450 monooxygenase cDNA in tobacco was found to enhance the metabolism of phenylurea herbicides (Siminszky *et al.*, 1999).

Since phase I is a transformation step that prepares the xenobiotic to its further detoxification, the resulting product may not be less toxic than the parent compound. Furthermore, some xenobiotics may not need to undergo phase I reactions if they already possess the functional sites required for the subsequent conjugation reaction.

1.1.3. Detoxification: phase II

In phase II, phase I-activated compounds are covalently linked to endogenous, hydrophilic molecules such as glucose, malonic acid, amino acids or glutathione. This conjugation step results in a more hydrophilic compound which is less likely to partition through biological membranes, and hence generally exhibits a decreased toxicity. Consequently, phase II reactions are generally protective although some conjugated xenobiotics retain some toxicity (Monks *et al.*, 1990). In plants, the principal enzymes involved in the conjugation of these endogenous molecules are glutathione-*S*-transferases, glucosyltransferases and malonyltransferases.

1.1.3.1. Glutathione S-transferases

Glutathione *S*-transferases (GSTs) are amongst the best-characterised enzymes involved in xenobiotic metabolism. They were first discovered in plants for their role in the detoxification of the herbicide atrazine in maize (Frear and Swanson, 1970). Since, their involvement in herbicide selectivity in both crops and weeds has been well-established (Cole *et al.*, 1997).

GSTs are cytosolic, soluble, dimeric proteins with a native molecular weight of approximately 50 kDa, which have been found in bacteria, fungi, plants and mammals (Sheehan *et al.*, 2001) but membrane-bound proteins with GST-like activities have also been reported (Jakobsson *et al.*, 1999). Generally, GSTs catalyse the transfer of the tri-peptide glutathione (γ-glutamyl-cysteinyl-glycine, GSH), in its thiolate anion form GS^-, to xenobiotics which possess a suitable electrophilic centre (equation 1.2). However, in some legumes such as mung bean and soybean, homoglutathione (γ-glutamyl-cysteinyl-β-alanine, hGSH) replaces glutathione and thus, hGSH-conjugates are formed (Coleman *et al.*, 1997).

$$GS^- + X^{\delta+} \xrightarrow{GST} GS\text{-}X \qquad \text{(Equation 1.2)}$$

GSTs consist of two polypeptide subunits of approximately 26 kDa linked by a 5-10 residue linker, and exist as homodimers or heterodimers. Each subunit possesses a catalytic site that includes: (1) a well-conserved GSH-binding site in the *N*-terminal domain and (2) a more variable substrate-binding site in the C-terminal domain, which might explain the wide substrate specificity of this enzyme superfamily. Indeed, apart from the well-characterised role in pesticide detoxification, physiological functions of GSTs include:

- protection from oxidative stress, as they can act as glutathione peroxidases (Noctor and Foyer, 1998; Cummins *et al.*, 1999);
- isomerisation of catabolites during the degradation of aromatic amino acids (Fernandez-Canon and Penalva, 1998);
- and non-catalytic roles such as the binding, transport and compartmentation of phytohormones and phenolic compounds (Marrs, 1996; Alfenito *et al.*, 1998; Edwards *et al.*, 2000).

GSTs were first identified in mammalian systems and were classified into alpha, mu, pi, sigma and theta classes on the basis of amino acid identity, immunological

cross-reactivity and substrate specificity. Most plant GSTs are similar to the theta class but were originally classified into three subgroups (I, II and III), according to their amino acid sequence identity and their number of introns and exons. Following the completion of the *Arabidopsis* genome sequencing project, which revealed that the *Arabidopsis* genome contains 48 GST-like genes (Dixon *et al.*, 2002), a new classification for plant GST genes was proposed recently (summarised in table 1.1). This classification is based on phylogenetic information and modelled from the non-mammalian Greek-letter system (Edwards *et al.*, 2000).

Interestingly, not all enzyme activities documented to date are sub-class specific, for example, glutathione peroxidase activity is found in theta, phi and tau class members. Although GSTs of different subclasses have low overall sequence identity, three-dimensional structures obtained by X-ray crystallography demonstrated a highly conserved overall structure (Sheehan *et al.*, 2001) but with subtle differences in the active site, which often provide a structural rationale for substrate specificity (Thom *et al.*, 2001; Thom *et al.*, 2002).

1.1.3.2. Glucosyltransferases

Glycosyltransferases are a group of enzymes that catalyse the transfer of an activated mono- or oligosaccharide residue to an acceptor molecule. They have been detected in a wide variety of organisms, ranging from mammals to micro-organisms (bacteria, archaea and fungi). In plants, UDP-glucose is the most common glycosylation substrate and is the substrate for *O*- and *N*-glucosyltransferases (equations 1.3 and 1.4).

$$\text{R-OH} + \text{UDP-glucose} \xrightarrow{\textit{O}\text{-GT}} \text{R-O-glucose} + \text{UDP} \qquad \text{(Equation 1.3)}$$

$$\text{R-NH}_2 + \text{UDP-glucose} \xrightarrow{\textit{N}\text{-GT}} \text{R-NH-glucose} + \text{UDP} \qquad \text{(Equation 1.4)}$$

Glucosyltransferases (GTs) involved in xenobiotic detoxification are soluble, cytosolic enzymes, with molecular masses between 45 kDa and 60 kDa though membrane-bound GTs involved in secondary metabolism have been detected in the Golgi apparatus and the endoplasmic reticulum (Winkel-Shirley, 1999). GTs play major roles in a range of plant processes:

- plant hormone homeostasis, e.g. the conjugation of the phytohormone indole-3-acetic acid (Jones and Vogt, 2001);
- cell-wall biosynthesis, e.g. in the phenylpropanoid pathway for the formation of lignin (Keegstra and Raikhel, 2001);
- plant defence and stress tolerance, e.g. the glucosylation of the signal molecule salicylic acid (Jones and Vogt, 2001);
- the detoxification of xenobiotic compounds such as pesticide derivatives (Sandermann *et al.*, 1997).

The addition of a sugar moiety to these molecules not only results in increased water solubility and improved chemical stability due to the decrease in reactivity, but also seems to act as a recognition signal for vacuolar sequestration, since glucosyl-conjugates are usually recognised by active transport systems, while their aglycones (non-carbohydrate portion of a glycoside) are not.

In plants, the glucosylation of pesticides during their detoxification process has been widely observed (Sandermann *et al.*, 1997). Because many xenobiotics are hydroxylated in plants, *O*-glucosides are the conjugates most frequently found (Pflugmacher and Sandermann, 1998). However, the formation of ester conjugates (by –COOH substitution) is readily reversible and therefore does not constitute an effective detoxification step. These labile molecules can nevertheless be further conjugated to more glucose residues to yield di- or oligo-saccharides, which may eventually be incorporated into the cell wall structure as bound residues (Schmidt *et al.*, 1997). By contrast, the *N*-glucosylation (transfer of a glucosyl-residue onto an amino group) of toxic compounds leads to conjugates which are generally more stable to enzymatic hydrolysis but that are labile to chemical hydrolysis at extreme pH values

New classification	Previous classification	Specific features	Functions
Phi	Type I	Three exons	Ligandin Isomerase GST detoxification GPOX detoxification
Zeta	Type II	Ten exons, close to mammalian zeta GSTs	Isomerase MAAI
Tau	Type III	Two exons	Hormone-responsive Ligandin GST detoxification GPOX detoxification
Theta	Type IV	Similar to mammalian theta GSTs	GPOX detoxification

Table 1.1: New classification of plant glutathione *S*-transferases.

After Edwards *et al.*, 2000.

Abbreviations: GST: glutathione *S*-transferase; GPOX: glutathione peroxidase; MAAI: maleylacetoacetate isomerase

(Winkler and Sandermann, 1989). Consequently, the metabolism of herbicides via the action of *N*-GTs is believed to be more efficient. The occurrence of other, less common glucosyl-conjugates, such as N-, S- and ester-glucosides also exist but will no be dealt with here. For further information, the reader is referred to Cole and Edwards (2000).

Until the mid-1990s, very little information regarding the sequence and structure of glycosyltransferases was known and, due to the wide range of sugar substrates and acceptors involved, a simple and obvious classification of glycosyltransferases was impossible. Many GTs had been purified from various plant species, mammals and micro-organisms but only a single three-dimensional structure of a glycosyltransferase, a DNA β-glucosyltransferase from bacteriophage T4 was available (Vrielink *et al.*, 1994). In 1997, Campbell and colleagues proposed a first classification for NDP-sugar hexosyltransferases (EC 2.4.1.*x*), based on amino acid sequences. According to this classification, there are now 65 different families of glycosyltransferases (http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html). However, with the recent access to the *Arabidopsis* genome sequence and the availability of 13 new crystal structures, new classification schemes have emerged. Kapitonov and Yu (1999) suggested the existence of three main families, based on the configuration of the anomeric carbon and conserved domains, whereas Ünligil and Rini (2000) classified glycosyltransferases according to their conserved catalytic domains. This classification into two superfamilies: GT-A, which comprises many Golgi apparatus- and endoplasmic reticulum-bound enzymes as well as many prokaryotic glycosyltransferases, and GT-B, which includes most of the prokaryotic enzymes involved in the glycosylation of primary and secondary metabolites, has since been adopted (Hu and Walker, 2002). The striking feature about glycosyltransferases is their structural similarities despite their very low sequence homology. These new discoveries allow more studies on enzyme mechanisms and structure-function relationships (Breton and Imberty, 1999; Breton *et al.*, 2001) and help clarify the functions of the gene products (Charnock *et al.*, 2001). 107 sequences in the genome of *Arabidopsis thaliana* contain a consensus suggesting that they belong to GT-A superfamily (Bowles, 2002). No such information was found for the GT-B superfamily.

1.1.3.3. Malonyltransferases

Malonyltransferases (MTs) catalyse the transfer of a malonyl group from malonyl-coenzyme A (M-CoA) to either a hydroxyl- or an amino-group of an acceptor molecule and are classified as *O*-MTs and *N*-MTs, respectively (equations 1.5 and 1.6).

$$\text{R-OH + M-CoA} \xrightarrow{\textit{O}\text{-MT}} \text{R-O-malonyl + CoASH} \qquad \text{(Equation 1.5)}$$

$$\text{R-NH}_2\text{ + M-CoA} \xrightarrow{\textit{N}\text{-MT}} \text{R-NH-malonyl + CoASH} \qquad \text{(Equation 1.6)}$$

Many flavonoids, found as 6'-malonylglucosides, have been detected within the vacuole (Matern *et al.*, 1986; Mackenbrock *et al.*, 1992; Mackenbrock *et al.*, 1993). Some anthocyanins (Harborne, 1986), amino acids (Guo *et al.*, 1993; Wu *et al.*, 1995), degradation metabolites of chlorophyll (Hinder *et al.*, 1996; Hortensteiner, 1998) and of 2,4-dichlorophenoxyacetic acid (Schmitt and Sandermann, 1982) are malonylated and deposited in the vacuole. These observations suggest that, in the same manner as glutathionation and glucosylation, malonylation could act as a signal for compartmentation (Matern *et al.*, 1984; Schmitt *et al.*, 1985; Schmidt *et al.*, 1997). However, in contrast to GSTs and glycosyltransferases, very little is known about MTs. Only *O*-MTs and their role in plant xenobiotic detoxification and secondary metabolism will be described in this section; *N*-MTs, which is the enzyme class studied in this thesis, will be discussed more in depth in a later paragraph.

O-MTs transfer a malonyl-residue to the 6'-hydroxy-group of a sugar moiety, generally glucose. This malonylation has been shown to protect glucosides from degradation by β-glucosidases (Suzuki *et al.*, 2002). However, the resulting malonylated glucosides are often labile and readily degraded under alkaline conditions or due to the action of malonylesterases and β-glucosidases (Schmitt *et al.*, 1985; Parry *et al.*, 1994). Due to the instability of this bond and the utilisation of inappropriate

extraction procedures, many 6''-*O*-malonylglucosides may often have been overlooked (Matern *et al.*, 1983; Harborne, 1986).

O-MTs have been shown to be involved in the metabolism of the herbicide methazole (Suzuki and Casida, 1981), the fungicide pentachlorophenol (Scheel *et al.*, 1984; Schmitt *et al.*, 1985) and 4-nitrophenol, which is the degradation product of pesticides such as parathion and fluorodifen (Schmidt *et al.*, 1993). They are also involved in the biosynthesis of phenolic compounds and have been purified from various sources. Two *O*-MTs specific for flavones were purified from parsley cell suspension cultures (Matern *et al.*, 1983) and an *O*-MT specific for isoflavones, particularly biochanin A and formononetin, was purified from chick pea roots (Koester *et al.*, 1984). More recently, *O*-MTs involved in the malonylation of anthocyanins were purified from the flowers of scarlet sage (Suzuki *et al.*, 2001) and dahlia (Suzuki *et al.*, 2002). cDNAS encoding these two enzymes were cloned, providing for the first time information regarding the molecular identity of malonyltransferases. Both *O*-MTs were approximately 50 kDa and possessed motifs commonly shared by members of the multifunctional plant acyltransferase superfamily (St-Pierre *et al.*, 1998), which led the authors to propose that *O*-MTs belong to this gene family. Members of the acyltransferase gene family differ considerably not only in their substrate but also in their co-substrate specificity. For example, many acyltransferases can accept both aliphatic and aromatic acyl donors, whereas *O*-MTs seem to accept only some aliphatic acyl donors, such as malonyl-CoA and succinyl-CoA but not acetyl-CoA (Bénichou *et al.*, 1995; Martin and Saftner, 1995). Acyltransferases have been shown to be involved in anthocyanin (Fujiwara *et al.*, 1998) and phenylpropanoid metabolism (Hoffmann *et al.*, 2003) but unlike the well-characterised GST and glycosyltransferase superfamilies, the functional and genetic characterisation of plant acyltransferases is only at its début.

It is unclear whether *O*-MTs involved in the metabolism of secondary products and of xenobiotics are identical. Matern and co-workers (1983) demonstrated in parsley cell cultures that partially purified malonyl-CoA: flavone/flavonol-7-*O*-glucoside malonyltransferase could use 4-hydroxy-2,5-D (a 2,4-D-derived metabolite) and 4-nitrophenol as substrates. However, when purified to homogeneity, the *O*-MTs did not

acylate these two compounds, suggesting that a contaminating *O*-MT was responsible for conjugating the xenobiotics.

1.1.4. Detoxification: phase III

In phase III, the xenobiotic-conjugate is compartmentalised either into the vacuole or into the apoplast, adding further protection against the xenobiotic, by physically removing it from its target-site, preventing potential feedback inhibition of phase II enzyme activities and permitting further metabolism in a discrete subcellular compartment.

1.1.4.1. Sequestration in the vacuole

The vacuole is the largest compartment in many types of plant cells and can occupy up to 90 % of a mature cell volume, e.g. in leaf mesophyll. It plays an important role as an intermediate storage compartment for carbohydrates, amino acids, organic acids and ions (reviewed by Martinoia *et al.*, 2000) and has a major protective role, since it is the accumulation site of xenobiotics and potentially toxic secondary metabolites (Wink, 1997).

The tonoplast (vacuolar membrane) possesses three major protein components: aquaporins (water channels), which regulate water volume and are crucial for osmotic movements, and two proton pumps (figure 1.1), the H^+-ATPase (V-ATPase) and H^+-pyrophosphatase (V-PPase). These two pumps maintain a proton gradient across the tonoplast (inside positively charged), and, together with the plasma membrane H^+-ATPase, contribute to the maintenance of cytosolic pH, such that the cytoplasmic pH stays around 7.4 (for a review, see Maeshima, 2001). The trans-tonoplast electrochemical gradient can be utilised by secondary transport systems such as symporters and antiporters (figure 1.1). Recently, a group of transporters that belong to the ATP-binding cassette (ABC) superfamily has been characterised (Rea *et al.*, 1998; Rea, 1999; Theodoulou, 2000). These transporters are found in both prokaryotes and eukaryotes and are involved in the active transport of a wide range of structurally

unrelated chemicals. All ABC-transporters possess a common basic structure consisting of two copies of two elements: a hydrophobic transmembrane spanning (TMS) domain, which consists of several membrane-spanning α-helices, and a cytosolic domain involved in ATP-binding, the nucleotide binding fold (NBF), which contains the Walker A and Walker B motifs and an ABC-transporter specific sequence (Walker *et al.*, 1982; Higgins, 1992). This structure allows these proteins to transport chemicals across biological membranes, via the hydrolysis of ATP. In fact, ABC-transporter catalysed uptake are characterised by a direct energisation by ATP hydrolysis, an extreme sensitivity to vanadate and are not dependent on the H^+-electrochemical gradient.

There are 13 subclasses of ABC proteins in plants (Sánchez-Fernández *et al.*, 2002), several of which might potentially be involved in transport of xenobiotics and their metabolites. In mammalian systems, members of at least two ABC-transporters have been shown to confer resistance to a range of structurally unrelated drugs and were hence called **m**ulti-**d**rug **r**esistance proteins (MDRs) and **m**ultidrug **r**esistance associated **p**roteins (MRPs). Representatives of these proteins are also present in plants and have since been shown to be involved in various aspects of xenobiotic transport. Of particular interest are members of the MRP subclass: they are involved not only in the vacuolar sequestration of negatively charged xenobiotic metabolites, such as glutathione-conjugates of herbicides, but also in the deposition of endogenous anionic compounds, such as glucuronides (Martinoia *et al.*, 1993; Klein *et al.*, 1998; Klein *et al.*, 2000; Gaedeke *et al.*, 2001; Klein *et al.*, 2001; Klein *et al.*, 2002). In contrast, secondary transport systems, generally H^+-antiport systems, have been implicated in the transport of both xenobiotic and endogenous glucosides, such as herbicide conjugates (Bartholomew *et al.*, 2002) and phenolic compounds, such as coumarins and flavonoids (Werner and Matile, 1985; Klein *et al.*, 1996; Bartholomew *et al.*, 2002).

1.1.4.2. Sequestration in the apoplast

The apoplast is generally defined as the continuous system of cell walls and intercellular air spaces of a plant. Using radiolabelled pesticides, metabolic fate experiments demonstrated that certain xenobiotics were ultimately found in the apoplast, possibly associated to pectin, cellulose, hemicellulose or lignin (Schmidt, 1999). Because these radiolabelled compounds cannot be extracted with common aqueous and organic solvents, they are referred to as non-extractable or bound residues. Various plant species (both mono- and dicotyledons) and systems (cell suspension cultures, intact plants, seeds, excised leaves/shoots) have been used to study the metabolic fate of a wide range of pesticides (reviewed by Schmidt, 1999). In general, considerable amounts of non-extractable residues were formed from heterocyclic and aromatic compounds (in particular anilines) whereas less bound residues resulted from the metabolism of linear, aliphatic compounds. The quantity of bound residues also varies greatly depending on the plant species, the tissues utilised and the growth conditions.

It is still unclear how pesticides, or their metabolites, are transported into the apoplast and incorporated into cell wall polymers. 3,4-dichloroaniline, a degradation product of many pesticides which is usually found as non-extractable compounds, was suggested to be bound to lignin via the α-carbon in the lignin side chain, thus forming a benzylamine bond (Still *et al.*, 1981). This was confirmed recently by NMR-spectroscopy (Brunow *et al.*, 1998; Lange *et al.*, 1998). The potential bioavailability of these compounds is also a matter for discussion. For instance, when radiolabelled 4-chloroaniline was applied to soil prior to the growth of barley, potatoes and carrots, approximately 30 % of the applied radioactivity was recovered from the plants after 20 weeks (Freitag *et al.*, 1984). This raised some concerns about their toxicology in the food chain (Sandermann *et al.*, 2001). Similarly, when chloroaniline-lignin conjugates obtained from wheat were fed to lambs and rats, 11-20 % of the radioactivity was released in a soluble form (Sandermann *et al.*, 1992). When enzymatically synthesised chloroaniline-lignin conjugates were used, a striking 66 % radioactivity was released from rats, as soluble metabolites. The same authors then simulated the acidic stomach

conditions of mammals (0.1 M HCl, 37 °C) and observed an immediate 30 % release of the bound residues. However, this was not confirmed *in vivo*. More recently, the degradation of chloroaniline-lignin conjugates under simulated stomach conditions and using NMR-spectroscopy showed that the previously reported high bioavailability was due to an experimental artefact (Lange *et al.*, 1998).

1.1.5. Detoxification: phase IV

The sequestration of toxic compounds into the vacuole or the apoplast appears to be an effective means of preventing cellular damage and, for a long time, this compartmentation was believed to be a final step in the detoxification of xenobiotics in plants. However, as illustrated by the potential bioavailability of bound residues, the metabolism of xenobiotics does not end with their sequestration. A well studied case is the metabolic fate of glutathione-conjugates, which are compartmentalised within the vacuole via ABC transporters (Martinoia *et al.*, 1993; Rea, 1999). As well as being an additional protection step, this compartmentation also prevents a possible feedback inhibition of GSTs by GSH-conjugates (Lamoureux and Rusness, 1986). However, once within the vacuole, GSH-conjugates are degraded by peptidases in a sequential fashion. First, a carboxypeptidase cleaves the glycinyl residue from the GSH moiety, forming a γ-glutamyl-cysteine-conjugate, which is subsequently degraded to an *S*-cysteinyl derivative. This conjugate is then believed to be exported into the cytosol where it undergoes malonylation, glucosylation or malonyl-glucosylation, or is further degraded to thiolactic acid, thiophenol and thioanisole derivatives (Schröder, 1997). These observations suggested the existence of an enzymatic network involved in the further metabolism of xenobiotics (Sandermann, 1994).

1.2. 3,4-Dichloroaniline, a model xenobiotic

3,4-Dichlororaniline (DCA) is the degradation metabolite of certain pesticides, such as Propanil (*N*-propionyl-3,4-dichloroaniline), Diuron [3-(3,4-dichlorophenyl)-1,1-dimethylurea] and Linuron [3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea] and also derives from the production of pharmaceuticals and azo-dyes. DCA production amounts to 42,000 to 47,000 tonnes worldwide, 60 % of which is produced in the EU (Livingston and Willacy, 1991).

DCA is a persistent pollutant in the environment as it binds covalently to soil particles, which limits its bioavailability to soil micro-organisms (Thorn *et al.*, 1996). Since DCA is not readily biodegraded, it accumulates in the soil following repeated use of certain herbicides. The metabolic fate of [^{14}C]-labelled DCA in sediments collected from different environmental sites, was studied by Heim and co-workers (1994) who demonstrated that DCA was predominantly associated to the sediments as non-extractable residues. DCA was nevertheless mobile in aqueous systems as it was released into the water surrounding the sediments, but was readsorbed within 15 to 25 d, by association to humic substances in the sediment (Heim *et al.*, 1995). However, DCA also appears to have a propensity for building-up within plant tissues. For example, it accumulates as bound-residues in wheat, soybean and carrot cell suspension cultures and plants (Winkler and Sandermann, 1989; Bockers *et al.*, 1994; Schmidt *et al.*, 1994). Propanil, a post-emergence herbicide used on rice (*Oriza sativa*) is hydrolysed to DCA, which is subsequently found as soluble and bound residues (Still, 1968; Yih *et al.*, 1968). As a consequence, phytoremediation is considered to be an attractive solution to DCA contamination (Schröder *et al.*, 2001; Schäffner *et al.*, 2002). In natural water, DCA is mainly degraded abiotically, by photolysis (Miller *et al.*, 1980; Othmen and Boule, 1999).

DCA degradation by micro-organisms and plants has been widely investigated. In contrast to plants, which use compartmentation as a means to detoxify xenobiotics, micro-organisms tend to export toxic compounds from the cells. Moreover, many micro-organisms can degrade aromatic compounds completely, a property that plants lack. Also, where plants use sugars and amino acids to conjugate xenobiotics, microbes

use non-nutrient endogenous substrates such as methyl and acyl residues from methanogenesis (for a review regarding the similarities and differences between microbial and plant xenobiotic detoxification, see Hall *et al.*, 2001). The metabolism of DCA by soil micro-organisms was reported to occur under anaerobic (Struijs and Rogers, 1989; Travkin *et al.*, 2002) and aerobic conditions (Surovtseva *et al.*, 1996) and led to the formation of organic by-products. Recently, El-Fantroussi (2000) demonstrated the complete mineralisation of urea herbicides by several bacterial species. But, because DCA is covalently bound to soil particles, its bacterial degradation is generally limited. However, the white-rot fungus *Phanerochaete chrysosporium* is capable of mineralising not only free chloroanilines but also those bound to the cell-wall (May *et al.*, 1997 and references therein). Consequently, the utilisation of *P. chrysosporium* may constitute an efficient remediation solution.

Like other chloroanilines, the primary human health hazard caused by DCA is its elicitation of methaemoglobin formation, which stops haemoglobulin from fixing oxygen reversibly (Singleton and Murphy, 1973). Anaemia and kidney and liver injury have also been observed following DCA poisoning (Valentovic *et al.*, 1995). Because of its toxicity and because it is not readily biodegradable, DCA has become one of the reference chemicals studied in eco-toxicological testing procedures and was chosen to be the model xenobiotic for this thesis. In plants, the metabolism of DCA has been studied using various species and systems, ranging from suspension-cultured cells to intact plants. The enzymes involved in DCA detoxification include *N*-glucosyltransferases and *O*- and *N*-malonyltransferases, as illustrated in figure 1.2.

1.3. *N*-malonyltransferases, current knowledge

N-malonyltransferases (*N*-MTs) are important for the detoxification of xenobiotics with free amino groups, such as DCA, which usually result from the phase I modifications of phenylurea herbicides such as Propanil, Diuron and Linuron. Malonylation has also been observed in the further metabolism of glucosides and GSH-conjugates. However, as opposed to the ester bonds formed following *O*-malonylations, the amide bonds formed by *N*-malonylations are more stable to chemical and enzymatic

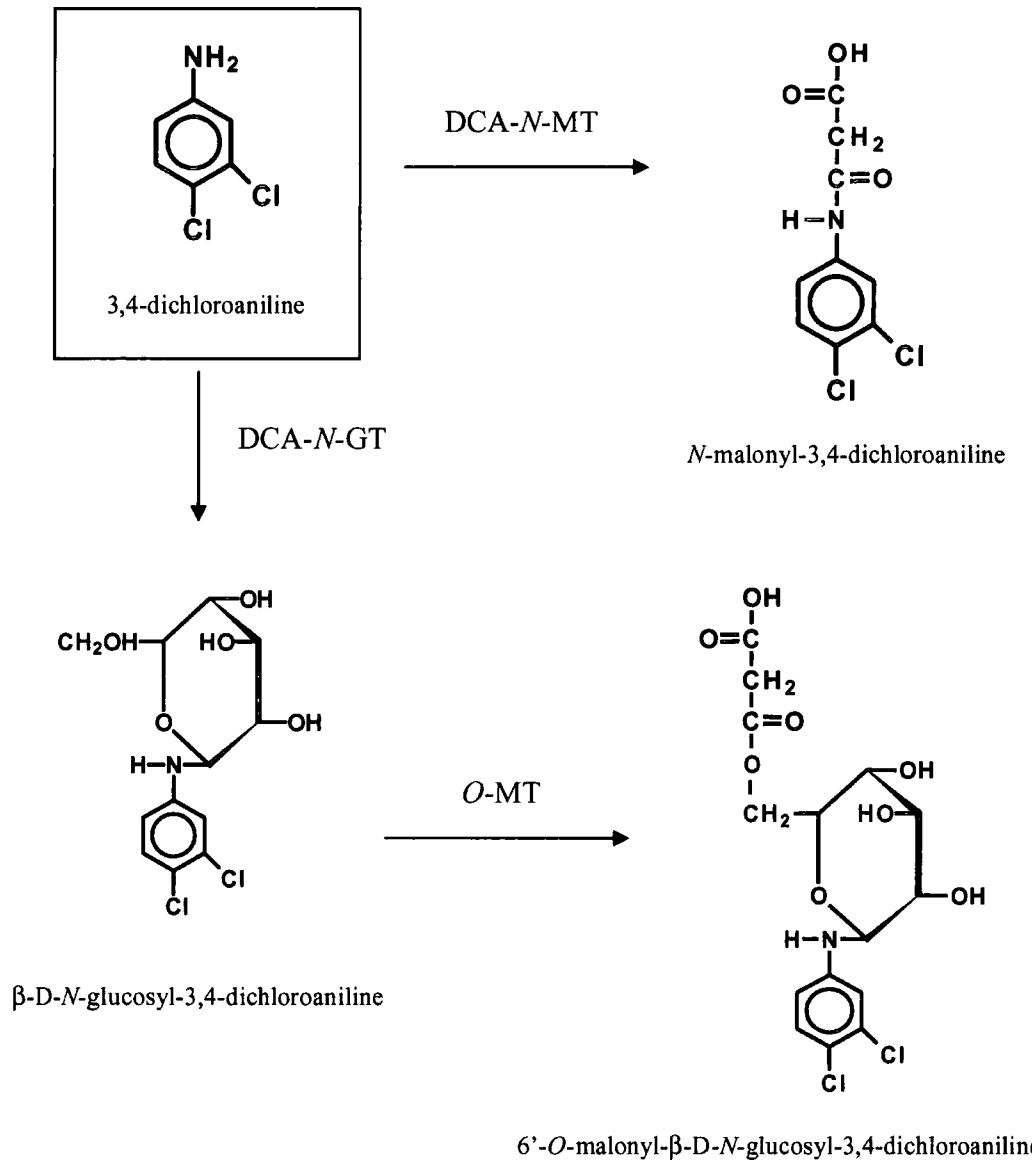


Figure 1.2: Enzymes involved in the metabolism of 3,4-dichloroaniline (DCA) in plants.

DCA-*N*-malonyltransferase (DCA-*N*-MT), DCA-*N*-glucosyltransferase (DCA-*N*-GT), *O*-malonyltransferase (*O*-MT).

hydrolysis (Winkler and Sandermann, 1989, 1992) and the resulting conjugates are generally compartmentalised, either in the vacuole or in the apoplast.

One of the best characterised roles of *N*-malonylation is in the conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) into its malonylated derivative (MACC), catalysed by ACC-*N*-MT (Kionka and Amrhein, 1984; Guo *et al.*, 1993). ACC being the immediate precursor of ethylene, its malonylation followed by its sequestration into vacuoles suggest that ACC-*N*-MT may contribute to the regulation of ethylene production (Yang and Hoffman, 1984). *N*-MTs may also be involved in the biosynthesis of auxins, as it has been suggested that *N*-malonyltryptophan is an auxin precursor and, since the activity of this trp-*N*-MT was increased by water-stress, *N*-MT may also be associated with stress response (Liu *et al.*, 1995; Wu *et al.*, 1995). *N*-MTs were additionally demonstrated to inactivate D-amino acids, which would otherwise be toxic to plants (Liu *et al.*, 1983; Guo *et al.*, 1993; Wu *et al.*, 1995).

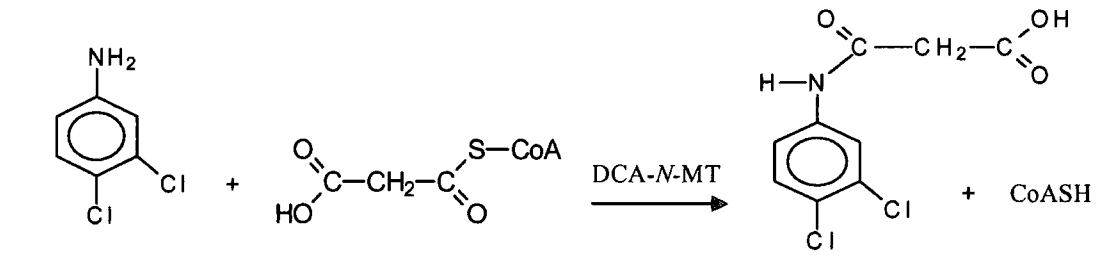
ACC-*N*-MTs and *N*-MTs accepting D-amino acids have been purified from various plant species (table 1.2). Some of these enzymes could only accept malonyl-CoA as acyl-donor, whereas others also accepted succinyl-CoA, though with lesser efficiency (40-50 % M-CoA activity; Bénichou *et al.*, 1995; Martin and Saftner, 1995). Some ACC-*N*-MTs appear to be substrate-specific whilst others accept both ACC and a range of amino acids. For example, neither a 54 kDa ACC-*N*-MT partially purified from chickpea nor a monomeric 38 kDa ACC-*N*-MT purified from tomato fruit (Martin and Saftner, 1995; Martinez-Reina *et al.*, 1996) were inhibited by L- and D-amino acids. In mung bean, three different monomeric *N*-MTs have been isolated. Firstly, a 55 kDa molecule was found to accept both ACC and D-phenylalanine and the enzyme activity was inhibited by aromatic amino acids (Guo *et al.*, 1992; Guo *et al.*, 1993) . Secondly, a 36 kDa *N*-MT which could also use both ACC and D-phenylalanine as substrates, was partially purified but unlike the 55 kDa protein, was not inhibited by aromatic amino acids (Benichou *et al.*, 1995). Thirdly, a 40 kDa ACC-*N*-MT was purified and used for monoclonal antibody production. The antibody recognised the enzyme at a site other than the active site and was capable of precipitating all ACC-*N*-MT activity present in mung bean crude extracts, suggesting the existence of several ACC-*N*-MT isoforms in mung bean (Chick and Leung, 1997).

Source enzyme	substrate specificity	MW (kDa)	pH optimum	Reference
peanut D-trp-*N*-MT	D-trp; also accepts ACC but with slow conversion	40	8.8	Matern *et al.*, 1984
peanut anthranilic acid-*N*-MT	anthranilic acid	50	8.8	Matern *et al.*, 1984
peanut DCA-*N*-MT	3,4-DCA	45	ND	Matern *et al.*, 1984
peanut DCA-*N*-MT	2-methoxyethanol	55	ND	Matern *et al.*, 1984
soybean DCA-*N*-MT	specific for chlorinated anilines (D-aas and ACC not tested)	48	6.3-7.2	Sandermann *et al.*, 1991
mung bean ACC-*N*-MT	ACC and D-phe; inhibited by aromatic amino acids	55		Guo *et al.*, 1992, 1993
mung bean ACC-*N*-MT	ACC and D-phe; accepts succinyl CoA	36		Bénichou *et al.*, 1995
tomato fruit ACC-*N*-MT	no inhibition by excess D-aa accepts succinyl CoA	38	8.0-8.5	Martin and Saftner, 1995
chickpea ACC-*N*-MT	no inhibition by D- or L- amino acids	54		Martinez-Reina *et al.*, 1996
mung bean ACC-*N*-MT		40		Chick and Leung, 1997

ND: not determined

Table 1.2: Properties of purified *N*-malonyltransferases.

Relatively little is known about *N*-MTs involved in the detoxification of xenobiotics. The metabolism of chlorinated anilines has been investigated mainly in wheat (*Triticum aestivum* L.) and in soybean (*Glycine max* L.), using a range of plant systems including cell suspension cultures (Scheel *et al.*, 1984; Schmitt *et al.*, 1985; Winkler and Sandermann, 1989; Sandermann *et al.*, 1991; Schmidt *et al.*, 1994), excised leaves (Gareis *et al.*, 1992) and whole plants (Bockers *et al.*, 1994; Höhl and Barz, 1995; Schmidt *et al.*, 1995). From these studies, the detoxification of DCA was assigned to a DCA-*N*-MT (EC 2.3.1.114), which transfers a malonyl-residue from M-CoA to the amino group of DCA (equation 1.7):



3,4-Dichloroaniline Malonyl-CoA M-DCA

(Equation 1.7)

So far, DCA-*N*-MT has been only partially purified from peanut seedlings (Matern *et al.*, 1984) and from soybean cell suspension cultures (Sandermann *et al.*, 1991). The enzymes were both monomeric and had molecular weights of 45 kDa and 48 kDa, respectively. The DCA-*N*-MT purified from soybean could metabolise a range of chlorinated anilines, with a clear preference for DCA, but had no activity towards natural products (Sandermann *et al.*, 1991). Unfortunately, ACC and D-amino acids were not tested as potential substrates in these studies. Interestingly, the purified 55 kDa ACC-*N*-MT from mung bean could not accept DCA but the latter was metabolised when crude mung bean extract was used, suggesting the existence of several substrate specific *N*-MTs in this plant species (Guo *et al.*, 1993). Although *N*-MTs are involved in secondary metabolism and play a role in the detoxification of herbicides, the

relationship between the enzymes involved in either of these functions is unknown. The molecular identification of DCA-*N*-MT will be an important step towards the understanding of the role and the classification of *N*-MTs in plants.

1.4. Aims

The overall aim of this project was to study soybean DCA-*N*-MT in order to obtain information which would lead to its molecular identification and to understand better the role of this enzyme in the detoxification of the reference xenobiotic DCA. Soybean was selected as a model dicotyledonous species since it is an important crop plant, is amenable to physiological, biochemical and molecular biological studies and since some information concerning DCA metabolism was already available.

Specific aims:

1. To study the metabolism of DCA in intact soybean plants with the aim of comparing data with previous results for other plant systems and extending these observations by examining the feasibility of DCA metabolite transport studies.
2. To establish a soybean cell suspension culture, since cell cultures have been shown to be a rich source of xenobiotic-degrading enzymes, including DCA-*N*-MT (Höhl and Barz, 1995; Schmidt *et al.*, 1995).
3. To devise a robust, rapid and sensitive assay for DCA-*N*-MT.
4. To compare DCA-*N*-MT in soybean plants and suspension cultured cells and to ascertain the best starting material for purification of DCA-*N*-MT.
5. To purify DCA-*N*-MT from soybean, with the ultimate aim of cloning and characterising the cDNA.

Chapter 2

Materials and methods

2.1. Chemicals

2.1.1. Tissue culture and biochemical studies

Chemicals for tissue culture were obtained from Sigma-Aldrich, England. Murashige and Skoog (MS) basal medium (M-5519); Schenk and Hildebrandt (SH) basal salt mixture (S-6765); 2,4-D (dichlorophenoxyacetic acid, D1133) and kinetin (6-furfurylaminopurine, K-0753).

Solvents for biochemical studies were of analytical grade. Malonic acid and *N,N'*-dicyclohexylcarbodiimide were obtained from Fluka, 3,4-dichloroaniline (DCA) from Riedel-de-Haën (Germany).

2.1.2. Radiochemicals

[UL-^{14}C]-3,4-dichloroaniline (22.1 mCi/mmol, 817.7 MBq/mmol) was obtained from Sigma-Aldrich (England), [2-^{14}C]-malonyl-Coenzyme A (55.0 mCi/mmol, 2.0 GBq/mmol) and Uridine diphospho-D-[UL-^{14}C]-glucose (320 mCi/mmol, 11.8 GBq/mmol) from Amersham Biosciences (England).

2.1.3. Synthesis of 3,4-dichloroaniline conjugates

N-malonyl-3,4-dichloroaniline (M-DCA) was synthesised chemically following the procedure described by Matern *et al.* (1984) for use as a reference compound in thin layer chromatography (TLC). Briefly, under anhydrous conditions, malonic acid and 3,4-dichloroaniline (DCA) were coupled in the presence of dicyclohexylcarbodiimide to form M-DCA and dicyclohexylurea. M-DCA was crystallized and the melting point was determined to be 134 °C, which is consistent with the results published by Matern *et al.* (1984).

Radiolabelled M-DCA and *N*-β-D-glucopyranosyl-3,4-dichloroaniline (G-DCA) were synthesised enzymatically using crude protein extracts (see section 2.4.1) of soybean suspension-cultured cells and *A. thaliana* suspension-cultured cells, respectively. For M-DCA, the assay mixture consisted of enzyme extract (100 µg protein), 8 mM DCA, 100 µM [2-^{14}C]-malonyl-Coenzyme A (0.5 µCi, 18.5 kBq) and reaction buffer (100 mM Bis-Tris Propane, pH 6.5 with HCl) in a final volume of 100 µl. After 20 min incubation at 35 °C, the reaction was stopped with 3 µl glacial acetic acid and the radiolabelled conjugates were extracted with 200 µl ethyl acetate. The ethyl acetate fraction was then analysed by thin layer chromatography (TLC, section 2.3.5) or used for radioactive quantitation by liquid scintillation counting (LSC, section 2.3.4). For G-DCA (protocol from C. Loutre, University of Durham), the assay mixture consisted of *Arabidopsis* root culture extract (50 µg protein), 60 µM DCA, 0.9 µM [UL-^{14}C]-UDP-glucose (2 nCi, 7.4 Bq) and reaction buffer (0.2 M Tris-HCl, pH = 8.0, 2 mM dithiothreitol). After 20 min incubation at 30 °C, the reaction was stopped with 125 µl reaction buffer and the radiolabelled conjugates were extracted with 200 µl ethyl acetate and analysed by TLC and LSC.

2.2. Plant material

2.2.1. Soybean tissue culture

Callus cultures from soybean (*Glycine max* L. var. Chapman) roots were established according to Schenk and Hildebrandt (1972) and Oswald *et al.* (1977). Briefly, the seeds were surface-sterilised [1 min in 70 % ethanol (v/v), 15 min in 8 % (w/v) sodium hypochlorite], rinsed 3 times for 10 min in distilled water, and placed onto Murashige and Skoog medium supplemented with 3 % (w/v) sucrose and 0.8 % (w/v) agar. The seeds were germinated in the dark at 26 °C, for 4 d. Root sections were placed onto "SH" medium, which consisted of Schenk and Hildebrandt (SH) basal salt mixture supplemented with 3 % (w/v) sucrose, 0.8 % (w/v) agar, 1 g l^{-1} *myo*-inositol, 5 mg l^{-1} thiamine, 5 mg l^{-1} nicotinic acid, 0.5 mg l^{-1} dichlorophenoxyacetic acid (2,4-D), 0.5 mg l^{-1} pyridoxine, and 0.1 mg l^{-1} kinetin, pH 5.8. The calli were grown at 26 °C, with 16 h photoperiod.

Friable cells growing on the surface of the calli were transferred into continuously-shaken 250 ml flasks containing 50 ml liquid SH medium (i.e. without agar). The use of baffled flasks to obtain finer suspension cultures was attempted but the cells did not survive the shear forces caused by the baffles. Therefore, small cell clumps were selected by pipetting during each sub-culture. Cells were sub-cultured every 2 weeks, initially by transferring 10 ml culture into 50 ml fresh medium but as the cells grew faster, 10 ml culture was transferred into 90 ml fresh medium (figure 2.1). The cultures were grown at 26 °C, with 16 h photoperiod.

2.2.2. Soybean

Soybean seeds (*Glycine max* L. var. Chapman) were sown in vermiculite and grown at 24 °C, with 110 μE m^{-1} s^{-1} light (16 h photoperiod), for 10 to 13 d. The seedlings were either utilised for the study of DCA metabolism (section 2.3) or for protein purification (section 2.4). For the study of DCA metabolism, the seedlings were transferred to Erlenmeyer flasks, each containing 50 ml Hoagland's No. 2 basal salt mixture (Sigma-Aldrich, England), pH 6.0 and incubated for 24 h with constant aeration to allow the plants to adapt to their liquid medium. Different treatments were applied to these seedlings, as described below (section 2.3).

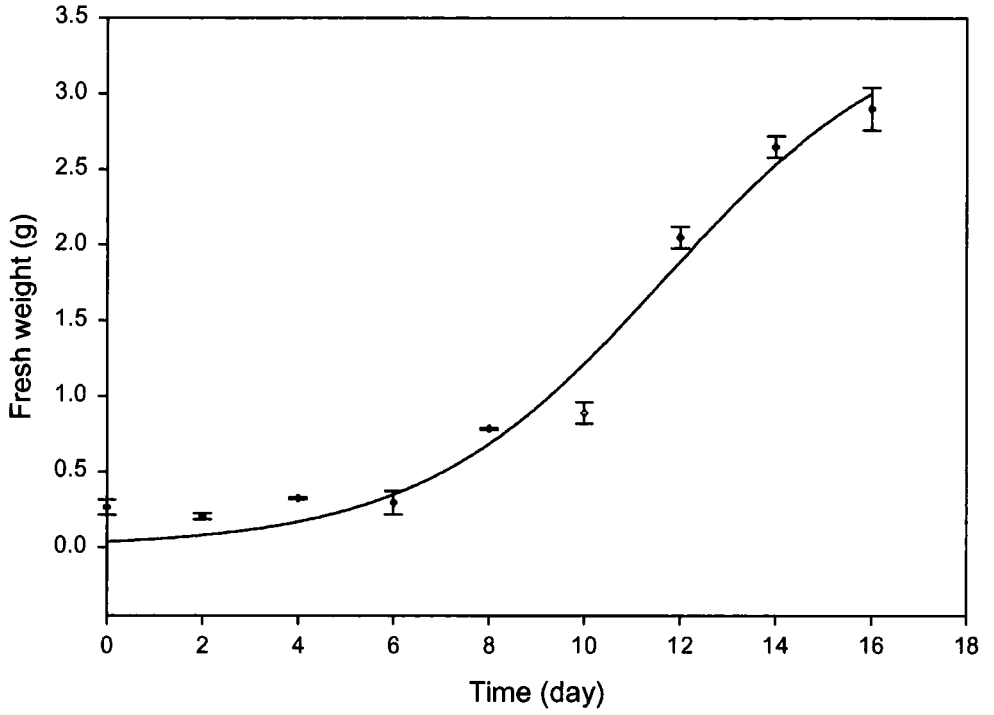


Figure 2.1: Growth curve for suspension-cultured soybean cells.

Cells were grown in Schenk and Hildebrandt (SH) medium, with 90 ml fresh SH medium inoculated with 10 ml two week-old culture. The fresh weight of cells was measured after vacuum-filtering the cells for 1 min, using a Büchner flask. Values are means ± standard deviation of four measurements.

2.2.3. Arabidopsis

For root cultures, the seeds of *Arabidopsis thaliana* wild type Columbia "Col 0" (Lehle Seeds, USA) were sterilised in 70 % (v/v) ethanol for 1 min before their transfer to a 20 % (v/v) sodium hypochlorite solution for 10 min. The seeds were then rinsed several times with sterile distilled water. Ten seeds were transferred to 50 ml Gamborg B5 liquid medium (Sigma-Aldrich, England), pH 5.8, supplemented with 1 % (w/v) sucrose and grown in the dark, at 25 °C with shaking (studies carried out in collaboration with C. Loutre, University of Durham). Cell suspension cultures (*Landsberg erecta*) were a gift from M. Fricker (University of Oxford; May and Leaver, 1993).

2.3. DCA metabolism studies

2.3.1. Treatment of soybean plants with DCA

9 d-old soybean plants were grown hydroponically (section 2.2.2) before DCA-treatment. After 24 h, [UL-^{14}C]-3,4-dichloroaniline (26.7 kBq, 0.7 μCi; final concentration 0.31 μM) was added to 6 flasks containing soybean seedlings, mixed thoroughly and left in the fume hood for 88 h, with the light on and with constant aeration of the media. To quantify and to identify DCA metabolites, the seedlings and the growth media were analysed separately as follows.

2.3.2. Extraction of radiolabelled DCA metabolites from plant material

To quantify the amount of radioactive DCA metabolites in various soybean organs, 2 batches of 3 seedlings were analysed for each treatment. The roots were rinsed first with tap water then with distilled water, blotted dry with tissues and each seedling was separated into roots (R), cotyledons (C), primary leaves (L), secondary leaves plus meristem (M), and stem (S). These were weighed, frozen in liquid nitrogen and ground

using a mortar and pestle, with acid washed sand. Methanol (10 v/w) at 4 °C was then added to extract the radiolabelled compounds. The mixture was transferred to 15 ml Falcon tubes, centrifuged at 4000 *g* for 5 min and the pellet frozen at -20 °C while the methanol supernatant was evaporated under pressure, using either a centrifugal evaporator at 50 °C (Programmable Centrifugal Evaporator RC 10.22, Jouan, England) or a rotary evaporator (Rotavapor EL130, Büchi, Switzerland). The dried material was resuspended in one ml methanol. The radioactive content of the samples was assessed by liquid scintillation counting (section 2.3.4) and the nature of the compounds was determined by High Performance Liquid Chromatography (HPLC, section 2.3.6).

Bound residues were solubilised from plant extracts by incubation with 2 ml of 2 M NaOH, at 37 °C for 20 h. The samples were neutralised with 320 µl concentrated HCl. One hundred µl of the supernatant was added to 4 ml Ultima-Gold High Flash Point Liquid Scintillation Counting cocktail (for aqueous and non-aqueous samples, Packard). Preliminary experiments showed that the amount of salt solution utilised and the variation of sample pH (from pH 1.6 to 11.1) affected the radioactive readings by ≤ 2 %.

2.3.3. Extraction of DCA metabolites from growth media

Samples from each flask of growth medium were counted by LSC. The three nutrient solutions from the same batch were then mixed together and the resulting 50 ml solution added to 50 ml ethyl acetate to partition the radiolabelled chemicals into the organic phase. The ethyl acetate phase was then concentrated to 1 ml under pressure, at 40 °C. Radioactivity in the distillate was quantified by LSC. Losses of volatile radioactivity by this procedure were typically 40 %. 200 µl of the liquid phase and 20 µl of the concentrated ethyl acetate were counted by LSC.

2.3.4. Liquid Scintillation Counting (LSC)

2.3.4.1. Quench curve

A quench curve was required to compensate the loss of counts due to the presence of ethyl acetate. To do so, a known amount of radioactivity was counted before and after the addition of increasing volumes of ethyl acetate (0 to 800 µl) to determine the quench produced. In practice, the radioactivity present in a series of 6 vials, each containing: 21,650 dpm of [^{14}C]-hexadecane (25 µl) and 3.0 ml Ultima-Gold^{TM} High Flash Point LSC cocktail for aqueous and non-aqueous samples (Packard), was measured on a Tri-Carb® Liquid Scintillation Analyzer (Packard) for 30 min, before and after the addition of increasing amounts of ethyl acetate (0, 50, 100, 200, 400 and 800 µl) into the vials to assess the quench produced. The quench curve was then plotted as the quench indicating parameter tSIE (transformed Spectral Index of the External standard) versus efficiency (figure 2.2). tSIE is equal to 1000 for an unquenched sample. As the counting efficiency decreases, due to increasing quench in the samples, the tSIE will also decreases. Therefore, by calculating the tSIE of a sample, the counting efficiency of that sample can be interpolated from the quench curve and the DPM (disintegration per minute) can be calculated as DPM = CPM/efficiency, with CPM: counts per minute.

2.3.4.2. Counting of samples

For the studies on the metabolism of [UL-^{14}C]-DCA in soybean plants (sections 2.3.2 and 2.3.3), 20 µl ethyl acetate extract was mixed with 4 ml of scintillation cocktail Ecoscint ™ A (National Diagnostics) or with 3.0 ml Ultima-Gold^{TM} LSC cocktail before quantification by LSC. The results obtained were similar. For DCA-*N*-MT assays, 100 µl ethyl acetate was mixed with 3.0 ml Ultima-Gold^{TM} LSC cocktail. The amount of radioactivity was measured on a Tri-Carb® Liquid Scintillation Analyzer (Packard) for 3 min.

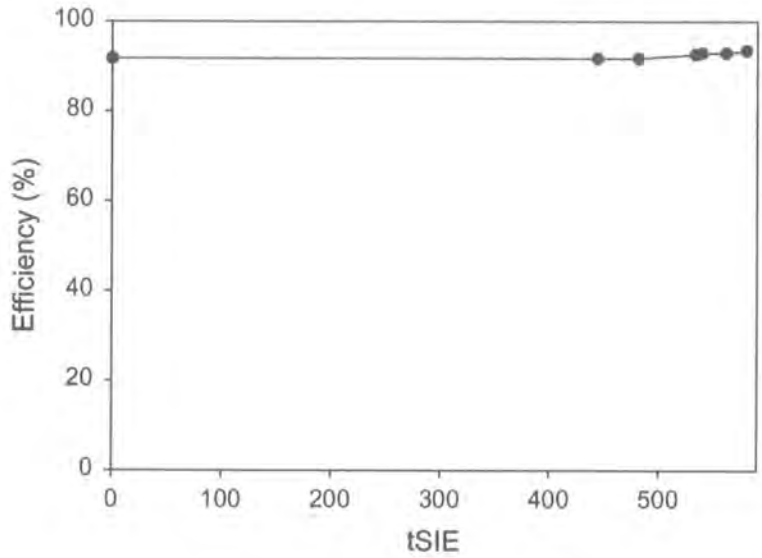


Figure 2.2: Ethyl acetate quench correction for liquid scintillation analyser.
The quench curve was constructed using a series of 6 vials, each containing: 21,650 dpm of [^{14}C]-hexadecane (25 µl) and 3.0 ml LSC cocktail. The radioactivity present in the vials was measured on a Tri-Carb® Liquid Scintillation Analyzer (Packard) for 30 min, before and after the addition of increasing amounts of ethyl acetate (0, 50, 100, 200, 400 and 800 µl) into the vials to assess the quench produced. tSIE: transformed Spectral Index of the External standard, is a parameter that indicates the level of quench in samples. For a completely unquenched sample, tSIE is equal to 1000.

2.3.5. Thin layer chromatography (TLC)

To analyse the radiolabelled conjugates synthesised enzymatically (section 2.1.3), approximately 20 µl (0.17 kBq) of ethyl acetate extract was spotted onto silica gel TLC plates pre-coated with fluorescent indicator (UV254, Sigma-Aldrich, England). The compounds were separated in solvent mix A [ethyl acetate/propan-2-ol/water (63/23/11, v/v/v)]. The Rf values for DCA, G-DCA and M-DCA were 0.78, 0.56 and 0.34, respectively. Radioactivity was detected and quantified using a TLC analyser (Berthold LB 2842, Wildbad, Germany). UV-absorbing spots were detected under UV light (254 nm).

In order to analyse DCA metabolites present in Hoagland's medium (after incubation of soybean plants with [UL-^{14}C]-DCA), solvent mix A or B [chloroform/methanol/water (60/35/8, v/v/v)] was utilised. With the latter, the Rf values for DCA, G-DCA and M-DCA were 0.76, 0.49 and 0. 44, respectively.

Radioactivity was detected using a phosphor-imager (Bio-Rad GS-525 Sample loading doc, Molecular Imager System and Screen eraser, England). Alternatively, radioactive spots were detected using Kodak BioMax TranScreen intensifying screen system (Kodak Scientific Imaging Systems, Cambridge, England), by placing the phosphor surface of the TranScreen between the TLC plate and the X-ray film (Kodak BioMax MR film) to increase the resolution of the signal detected. In both cases, identity of the conjugate was confirmed by co-chromatography with chemically-synthesised DCA conjugates (section 2.1.3).

2.3.6. High Performance Liquid Chromatography (HPLC)

The radiolabelled compounds extracted in ethyl acetate were evaporated using a rotary evaporator under pressure, at 50 °C and the residue redissolved in methanol (analytical grade). The plant and medium extracts were then analysed on a reverse-phase column (Symmetry C18, 3.5 µm, 4.6 x 30 mm). The initial solvent system of acetonitrile (5%)/1% phosphoric acid (95%) was changed after 2 ml to give a linear gradient to 100% acetonitrile at 7.6 ml, with a flow rate of 0.8 ml/min. The UV detector

was set at a wavelength of 254 nm. Reference standards: [UL-^{14}C]-G-DCA, G-DCA, DCA, [2-^{14}C]-M-DCA and M-DCA (sections 2.1.2 and 2.1.3).

2.3.7. Visualisation of DCA metabolites by phosphor-imaging

[UL-^{14}C]-3,4-dichloroaniline (37 kBq, 1 μCi; final concentration, 0.43 μM) was added to each flask containing 10 d-old soybean seedlings, mixed thoroughly and left at room temperature for 1 d, 3 d and 5 d. At each time-point, the seedling was rinsed with distilled water, blotted dry and pressed between two sheets of filter paper until dried before being analysed on a phosphor-imager (BioRad GS-525).

2.3.8. DCA-*N*-MT assay

2.3.8.1. Initial DCA-N-MT assay

The reaction was performed in 100 mM potassium phosphate buffer, pH 6.5, with 205 μM cold DCA, 19 μM [2-^{14}C]-M-CoA (7.4 kBq, 200 nCi) and enzyme extract, to a final volume of 200 μl. The reaction was incubated at 40 °C for 40 min, stopped with the addition of 5 μl glacial acetic acid and the radioactive conjugate was extracted with 200 μl ethyl acetate. The compounds were then separated by TLC and radioactive spots were detected and quantified by a linear TLC analyser (Berthold LB2842, Wilbad, Germany).

2.3.8.2. Optimised DCA-N-MT assay

The development and optimisation of the assay is fully described in Chapter 4. The assay mixture consisted of 100 mM Bis-Tris propane (pH 6.5 with HCl), 300 μM DCA, 19 μM [2-^{14}C]-Malonyl-CoA (7.4 kBq, 200 nCi), 0.1 % (w/v) BSA and 10-20 μg of protein extract, in a final volume of 100 μl. Radiolabelled M-CoA was added after pre-incubation of the reaction mixture at 35 °C for 2 min. The reaction was stopped

with 3 µl glacial acetic acid after 5 min incubation and the malonylated conjugates were extracted with 200 µl ethyl acetate, which was analysed by LSC (section 2.3.4).

2.3.8.3. Final DCA-N-MT assay

In order to limit the cost of the assay, the radiolabelled M-CoA used in the optimised assay was partially replaced with unlabelled M-CoA. Thus, 9 µM [2-^{14}C]-Malonyl-CoA (3.7 kBq, 100 nCi) was mixed with 22 µM unlabelled M-CoA prior to their addition to the reaction mixture, as described in 2.3.8.2. Again, to limit the cost of the assay, fractions eluted from chromatography columns were assayed with only 4 µM [2-^{14}C]-Malonyl-CoA.

2.3.9. *N*-Glucosyltransferase assay

The assay mixture consisted of 200 mM Tris-HCl, (pH 8.0), 1 mM DCA, 1 µM [UL-^{14}C]-UDP-glucose (26 nCi, 0.96 kBq) and 100 µg of crude protein extract, in a final volume of 75 µl. After incubation at 30 °C for 20 min, the reaction was stopped with 125 µl of 200 mM Tris-HCl, (pH 8.0) and the glucosylated conjugates were extracted with 200 µl ethyl acetate, which was analysed by LSC (section 2.3.4).

2.3.10. Assay for the presence of 6''-*O*-malonyl-DCA-*N*-(β-D-glucopyranosyl)-3,4-dicloroaniline (M-G-DCA)

To determine whether the DCA-glucosylated metabolites extracted from the soybean seedlings were malonylated, the samples were treated with 0.2 M Tris to cleave the malonyl ester from the parent glucoside (Tiller *et al.*, 1994) and re-analysed by HPLC. Methanol was removed from the samples by rotary evaporation under pressure at 50 °C. 0.5 ml of 0.2 M Tris was added to each extract and incubated 16 h at 30 °C. A control (sample incubated with distilled water) was included. After incubation, all samples were acidified with concentrated HCl to pH 7.0. The hydrophobic compounds

were subsequently extracted with 0.5 ml ethyl acetate, which was in turn evaporated and replaced with analytical grade methanol for analysis by HPLC (section 2.3.6).

2.3.11. Time-course of DCA uptake

16-d old soybean seedlings were transferred into 50 ml Hoagland's medium containing 37 kBq (1 μCi, final concentration, 0.86 μM) [UL-^{14}C]-DCA. Separate flasks were prepared for each time point. After 30 min, 2 h, 4 h, 8 h, 12 h, 24 h and 48 h, the plants and the media were analysed, as described in section 2.4, except that: (i) the plants were separated only into roots and shoots and the cotyledons (not radioactive) were discarded; (ii) the radiochemicals present in the growth media were extracted with one volume ethyl acetate; (iii) the methanol extracts were analysed by LSC (section 2.3.4) before and after concentration. All samples were in duplicate.

2.3.12. Cross-feeding experiment

Four soybean seedlings and four *A. thaliana* root cultures were transferred respectively into 50 ml Hoagland's medium and Gamborg B5 basal salt medium, containing 37 kBq (1 μCi) [UL-^{14}C]-DCA. After 48 h, plants were removed and the radiolabelled metabolites present in the growth media were assayed by LSC and then extracted by partitioning with 1 vol. ethyl acetate. The organic phase was evaporated under pressure using a rotary evaporator and the residue redissolved in 1 ml of methanol and re-assayed by LSC. After concentrating the methanolic extract to 50 μl under a stream of N_2, the equivalent of 6.67 kBq (final concentration 0.39 μM) of ^{14}C-labelled metabolites was added to 20 ml of fresh media in the following combinations: (1) fresh soybean plants plus DCA metabolites from soybean treatments (2) fresh *Arabidopsis* root cultures plus DCA metabolites from soybean treatments (3) fresh soybean plants plus DCA metabolites from *Arabidopsis* treatments (4) fresh *Arabidopsis* root cultures plus DCA metabolites from *Arabidopsis* treatments. After 24 h, the plant material and growth media were radioassayed as described above.

2.3.13. Effect of inhibitors on M-DCA efflux from soybean roots

12 d-old seedlings were grown overnight in Hoagland's (pH 6.0) before being transferred to a flask containing 50 ml fresh medium and 37 kBq (1 μCi) [UL-^{14}C]-DCA (3 plants/flask). After 30 min incubation at room temperature to allow the uptake of [UL-^{14}C]-DCA by the plants, the roots were thoroughly rinsed with distilled water, transferred to fresh media containing various inhibitors and left for 2 h. The seedlings were then thoroughly rinsed with distilled water, blotted dry, weighed and kept in liquid nitrogen for further use. The amount of radioactivity present in the media at the beginning of the experiment, after 30 min and after 2 h incubation with inhibitors, was quantified by LSC (section 2.3.4). The following inhibitors were used: 1 mM sodium vanadate, 1 mM sodium azide, 50 μM nifedipine, 100 μM quinidine, 10 mM butyric acid (pH = 5.6), 1 mM 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) and 1 mM probenecid. Nifedipine and quinidine were dissolved in DMSO. Control experiments showed that DMSO did not affect soybean roots in the amounts applied.

2.4. Purification of 3,4-dichloroaniline-DCA-*N*-malonyltransferase

A flow chart of the steps in the purification procedure is given in figure 2.3. All purification steps were carried out at 4 °C. Two strategies were employed, the first involved the use of Centricon filters and step-gradients for the elution of proteins from chromatographic columns whereas in the second, protamine sulphate was used and linear elution gradients were applied, with additional chromatographic steps.

2.4.1. Preparation of extracts

Step 1: Preparation of the crude extract. 100-500 g roots from 2 week-old soybean seedlings were washed thoroughly to remove vermiculite. The roots were then blotted dry before being frozen in liquid nitrogen and ground to a fine powder using a mortar and pestle. The powder obtained was mixed with 1.5 (v/w) homogenising buffer [200 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 5 % (w/v) PVPP, 5 mM DTT, 1 μg ml^{-1}

leupeptin, 1 μg ml^{-1} pepstatin A, 1 mM PMSF (added just before use, from a 0.2 M stock solution in ethanol)] and filtered through 4 layers of muslin. The filtrate was then cleared by centrifugation (20,000. *g*, 10 min) and the resulting supernatant was used as cell free extract for further purification.

2.4.2. Purification procedure 1

Step 2: Ammonium sulphate precipitation. Proteins were precipitated with finely powdered solid ammonium sulphate. Proteins that precipitated between 40-70 % salt saturation were collected after centrifugation (20,000 *g*, 20 min) and dissolved in suspension buffer (20 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 5 mM DTT, 1 mM PMSF). The mixture was then centrifuged at 10,000 *g* for 1 min to remove any insoluble material and was desalted using pre-packed Sephadex G-25 columns (PD-10, Amersham Biosciences, England), previously equilibrated with suspension buffer.

Step 3: Centricon filters. The proteins present in the desalted mixture were concentrated with Centricon filters (Millipore, UK). The extract was first filtered through a Centricon YM-100 (with 100 kDa cut-off). The filtrate obtained was then transferred onto a Centricon YM-30 (with a 30 kDa cut-off). Proteins retained by the last filter, with a theoretical size range between 30 and 100 kDa, were used for the following purification step.

Step 4: Ion-exchange chromatography. The protein mixture was applied to a Hi-Trap DEAE-Sepharose Fast Flow column (1.6 x 2.5 cm, Amersham Biosciences, England) at 2 ml min^{-1}, equilibrated with start buffer (20 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$) prior to use. Proteins were eluted using a step gradient of NaCl in the same buffer, incrementing by 50 mM NaCl with each step, from 0 to 450 mM NaCl. One ml fractions were collected and assayed for protein content and for DCA-*N*-MT activity. Ammonium sulphate [25 % (w/v)] was added to the active fractions to prepare for the following purification step.

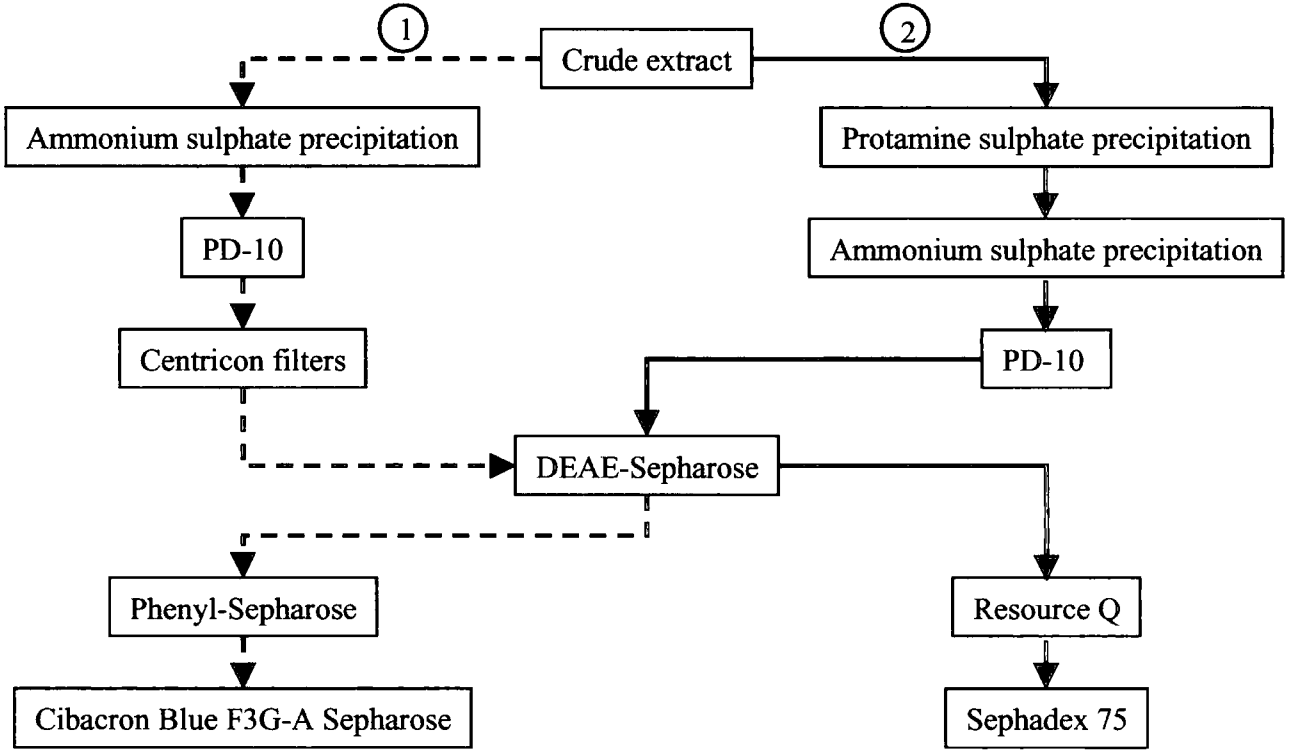


Figure 2.3: Strategies employed for the purification of DCA-*N*-MT from soybean roots.

DNA was precipitated using protamine sulphate and proteins were concentrated using ammonium sulphate. Different column chromatographies were utilised: PD-10 for desalting, DEAE-Sepharose and Resource Q as anion-exchange steps, Phenyl-Sepharose as a hydrophobic interaction step, Cibacron Blue as an affinity step and Sephadex 75 for size exclusion.

Step 5: Hydrophobic interaction chromatography. The protein concentrate was applied to a phenyl-Sepharose High Performance column (0.7 x 2.5 cm, Amersham Biosciences, England) at one ml min^{-1}, equilibrated with 25 % (w/v) ammonium sulphate in suspension buffer prior to use. Proteins were eluted using 2 ml step of 15 %, 10 %, 0 % ammonium sulphate in suspension buffer and double-distilled water. One ml fractions were collected and assayed for protein and enzymatic activity.

Step 6: Dye ligand chromatography. The active fraction was loaded on a HiTrap Cibacron Blue F3G-A-Sepharose High Performance column (1.6 x 2.5 cm, Amersham Biosciences, England) at 2 ml min^{-1}, equilibrated with start buffer prior to use. Proteins were eluted using a step gradient of NaCl in the same buffer, incrementing by 200 mM NaCl with each step, from 0 to 1 M NaCl. One ml fractions were collected and assayed for protein content and for DCA-*N*-MT activity.

2.4.3. Purification procedure 2

Step 2': Precipitation of nucleic acids. Nucleic acids were precipitated from the cell free extract using 0.14 % protamine sulphate [from a 1.4 % (w/v) protamine sulphate solution; protamine sulphate was first dissolved in concentrated HCl before being diluted further with distilled water]. After 15 min, the nucleic acid precipitate was removed by centrifugation (25,000 *g*, 45 min) and the supernatant was used for the next step.

Step 3': Ammonium sulphate precipitation. As described in step 2 above.

Step 4': Ion-exchange chromatography. The protein mixture was applied to a Hi-Trap DEAE-Sepharose Fast Flow column (1.6 x 2.5 cm, Amersham Biosciences, England), previously equilibrated with start buffer (20 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$). Proteins were eluted using a linear gradient of NaCl, up to 300 mM NaCl, at a flow rate of 2 ml min^{-1}, over 20 min. One ml fractions were collected and assayed for protein content and for DCA-*N*-MT activity. The active fractions were desalted using pre-packed Sephadex G-25 columns (PD-10, Amersham Biosciences,

England) and either loaded onto the next chromatography column or stored in liquid nitrogen.

Step 5': Second ion-exchange chromatography. The protein mixture was applied to a Resource Q column (16 x 30 mm, Amersham Biosciences, England), previously equilibrated with start buffer (20 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$). Proteins were eluted using a linear gradient of NaCl, from 0 to 1 M NaCl, at a flow rate of 3 ml min^{-1}, over a period of 30 min. 1.5 ml fractions were collected and assayed for protein content and for DCA-*N*-MT activity. The active fractions were desalted using pre-packed Sephadex G-25 columns (PD-10, Amersham Biosciences, England) and frozen in liquid nitrogen.

Step 5'': Gel filtration. In place of the second ion-exchange column, a size exclusion step was tested (HiPrep 16/60 Sephacryl S-100 High Resolution, 16 x 600 mm, Amersham Biosciences, England). The column was calibrated using albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), trypsin (20.1 kDa) and cytochrome c (12.4 kDa), with V0 = 37.4 ml (figure 4.16.A). All solutions were filtered through a cellulose nitrate membrane filter (0.2 µm pore size). The column was rinsed with 60 ml double-distilled water and 300 ml start buffer, at 0.5 ml min^{-1}, prior to the separation of protein samples.

2.4.4. Determination of protein concentration

2.4.4.1. Using a microtitre plate

The protein content of samples was determined by the method of Bradford (1976), adapted to a 96-well microtitre plate format. Briefly, a standard serial dilution of bovine serum albumin (BSA) was prepared by a series of two-fold dilutions, starting with 80 µl of a 0.2 mg ml^{-1} BSA solution in 80 µl of a 0.2 M potassium phosphate buffer, pH 7.2. Protein concentration was assessed by adding 20 µl of Bio-Rad Protein Assay Dye (ref. 500-0006) to each well, before mixing and incubating at room temperature for 5 min. Protein samples were treated identically. Both standards and samples were performed in triplicate. The absorbance at 595 nm

was measured in a microplate spectrophotometer system (SpectraMax™ 340, Molecular Devices, Sunnyvale, USA).

2.4.4.2. Using 1 ml cuvettes

The protein content of samples was determined by the method of Bradford (1976). A standard curve was prepared using 0.2 mg ml^{-1} BSA. 0, 10, 20, 40 and 80 µl of BSA solution was made up to 100 µl with distilled water and 900 µl of Bio-Rad Protein Assay Dye (diluted 1 in 5 prior to use) was added. The cuvettes were inverted to mix their content thoroughly and left at room temperature for 5 min before reading the absorbance at 595 nm. Samples were diluted so that their absorbance corresponded to the linear part of the standard curve. Both standards and samples were performed in duplicate.

2.4.5. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Polyacrylamide gel electophoresis of proteins was carried out using a Bio-Rad PROTEAN minigel kit according to manufacturers' instructions. SDS-PAGE gels consisted of a 12 % acrylamide lower resolving gel [1.75 ml 30 % (v/v) bis-acrylamide (37.5:1 acrylamide:bis), 1.75 ml 1.5 M Tris-HCl pH 8.8, 10 µl ammonium persulphate (250 mg ml^{-1}), 10 µl *NNN'N'*-tetramethylethylenediamine (TEMED), 30 µl 10 % (w/v) SDS, 3.45 ml distilled water], which was overlaid with water-saturated butanol and allowed to polymerise for ca. 30 min at room temperature. Once set, the water-saturated butanol was poured off and the gel was rinsed with distilled water and dried with filter paper. The stacking gel [0.5 ml 30 % (v/v) bis-acrylamide (37.5:1 acrylamide:bis), 0.9 ml 0.5 M Tris-HCl pH 6.8, 5.1 µl ammonium persulphate (250 mg/ml), 36 µl 10 % (w/v) SDS, 5.1 µl TEMED, 2.17 ml distilled water] was poured on top of the resolving gel, a comb was inserted to allow the formation of wells, and the gel was allowed to set at room temperature.

Protein samples were prepared by mixing with an equal volume of 2 x SDS sample buffer [5 % (v/v) glycerol, 3 % (w/v) SDS, 63 mM Tris-HCl pH 6.8, 25 mg bromophenol blue, 5 % (v/v) β-mercaptoethanol (14.3 M), made up to a final

volume of 50 ml with distilled water], and boiled for 10 min. Samples were loaded into the wells and electrophoresed for ca. 45 min at 200 V in gel running buffer [25 mM Tris-HCl pH 8.3, 250 mM glycine, 0.1 % (w/v) SDS]. To visualise the bands, gels were stained for 1 h in staining buffer [40 % (v/v) methanol, 20 % (v/v) acetic acid, 0.2 % (v/v) Coomassie Brilliant Blue R-250] and destained in 30 % (v/v) methanol, 10 % (v/v) acetic acid until bands became visible.

2.5. Further characterisation of DCA-*N*-MT

2.5.1. Effect of DCA on DCA-*N*-MT activity

7 d-old soybean seedlings, grown as described in section 2.2.2 (except at 26 °C instead of 24 °C), were transferred from vermiculite to 50 ml Hoagland's medium (pH = 6.0, 1 seedling per pot) and grown hydroponically overnight, with constant aeration, to allow the plants to adapt to the liquid medium. The volume of Hoagland's was re-adjusted to 50 ml with distilled water before the addition of 10 µl DCA stock solution, to give a final concentration of 100 µM and 200 µM DCA. DCA was added at 48 h, 24 h and 12 h before the determination of DCA-*N*-MT activity in the 10 d-old roots. A control, to which 10 µl methanol was added at each time point, was included. All samples were in duplicate. Protein extracts were obtained as described in section 2.4.1 and DCA-*N*-MT assay was performed as described in section 2.3.8.3, except that only 10 µg total protein was present in each assay.

A shorter time-course experiment was carried out in the same conditions, except that a final concentration of 100 µM DCA was applied to 15 plants. The DCA-*N*-MT activity was measured at 2 h, 4 h, 6 h and 12 h.

Similarly, soybean suspension-cultured cells were treated with 50 µM DCA and 100 µM DCA every 12 h for 36 h. Methanol was used as a control treatment. After 36 h, the 10 d-old cells were vacuum-filtered, weighed and frozen in liquid nitrogen. Proteins were extracted and DCA-*N*-MT assay performed as described above.

2.5.2. Kinetic studies

Eight, 9 d-old soybean seedlings, grown as described in section 2.2.2, were transferred from vermiculite to 150 ml Hoagland's medium (pH 6.0, 4 seedlings/150 ml) and grown hydroponically overnight to allow the plants to adapt to the liquid medium. The volume of Hoagland's was re-adjusted to 150 ml with distilled water before the addition of 30 µl DCA stock solution (final concentration of 100 µM DCA) or 30 µl methanol. After 24 h treatment, proteins were extracted as described in section 2.4., steps 1 and 2. DCA-*N*-MT assay was carried out as described in section 2.3.8.2, with the following modifications. To determine the K_m value for DCA, DCA concentration was varied from 0 to 600 µM. The assay included 3.7 kBq [2-^{14}C]-Malonyl-CoA and cold malonyl-CoA to give a final, constant concentration of 1 mM. To determine the K_m value for malonyl-CoA, DCA concentration was kept constant at 300 µM and the concentration of malonyl-CoA was varied from 0 to 1.2 mM (which consisted of 10 µM [2-^{14}C]-Malonyl-CoA and cold malonyl-CoA).

2.5.3. Effects of inhibitors on DCA-*N*-MT activity

Fourteen 9 d-old soybean seedlings, grown as described in section 2.2.2, were transferred from vermiculite to 30 ml Hoagland's medium (pH 6.0, 2 seedlings per 30 ml) and grown hydroponically overnight to allow the plants to adapt to the liquid medium. The medium volume was re-adjusted to 30 ml with distilled water before the addition of DCA, actinomycin D (actD) and cycloheximide (CH) in various combinations. Final concentrations for the treatments were: 100 µM DCA, 79.6 µM (100 µg ml^{-1}) actD and 35.5 µM (10 µg ml^{-1}) CH. They were applied either as sole treatments, or combined as follows: DCA + actD or DCA + CH. ActD was dissolved in absolute ethanol, therefore, a control to which 1.5 ml absolute ethanol was added was included. DCA was dissolved in methanol but previous experiments have shown that the volume utilised (< 1 ‰) had not effect on the growth of soybean. A negative control (distilled water) was also included. All treatments were for a period of 24 h and in duplicate. The roots were extensively rinsed in cold distilled water before being blotted dry, weighed and frozen in liquid nitrogen.

Protein extracts were obtained as described in section 2.4 and DCA-*N*-MT assay was performed as described in section 2.3.8, with 10 µg total protein instead of 20 µg.

2.5.4. *In vitro* translation of soybean RNA

2.5.4.1. RNA extraction and quantification

Total RNA was extracted from vermiculite grown 10 d-old soybean roots. 0.2 g root tissue was frozen in liquid nitrogen, ground with a mortar and pestle to a fine powder and mixed with 2 ml TRIzol® reagent (GIBCO Invitrogen Ltd, Paisley, Scotland) until thawed. The mixture was transferred to an autoclaved 2 ml microcentrifuge tube, vortexed for 20 s and incubated a room temperature for 5 min before being centrifuged (12,000 *g*, 10 min, 4 °C). The supernatant was transferred to a new tube, 0.4 ml chloroform was added and the tubes were vortexed for 10 s and incubated at room temperature for 5 min before another centrifugation (12,000. *g*, 15 min, 4 °C). At this stage, the mixture separated into a lower pink-phenol-chloroform phase and a colourless upper aqueous phase, which contained the RNAs. The upper phase was transferred to a new tube, mixed with 0.6 vol. isopropanol and 0.1 vol. DEPC-treated 3 M sodium acetate, incubated at room temperature for 10 min before being centrifuged (12.000 *g*, 10 min, 4 °C) to pellet the RNAs. The supernatant was then removed with a pipette and the pellet washed with 70 % (v/v) ethanol (made up with RNase free water). The tubes were centrifuged to re-pellet the RNAs (12,000 *g*, 5 min, 4 °C). The supernatant was removed and the pellets were air-dried for 10 min at room temperature and resuspended in 30 µl RNase free water.

RNA samples (diluted 1/100) were quantified by spectrophotometry at 260 nm and 280 nm. The concentration of RNA was then calculated as follows: [RNA] = $Abs_{(260\ nm)}$ x 40, in µg ml^{-1}. With the dilution factor of 100: [RNA] = $Abs_{(260nm)}$ x 4, in µg μl^{-1}. To assess the contamination of the samples with DNA, the ratio $Abs_{(260nm)}/Abs_{(280nm)}$ was calculated. For a pure RNA sample, this ratio equals 1.8.

The quality of RNAs was checked in a 1 % (w/v) agarose gel made up in TAE buffer (40 mM Tris-Acetate, 1 mM EDTA, pH = 8.0) with ethidium bromide (1 µl in 100 ml gel). 2 µl sample was mixed with 2 µl loading buffer [50 % (v/v) glycerol, 0.5 % Bromophenol blue, in RNase-free water] and 10 µl RNase-free

water to increase the total volume and facilitate the loading on the gel. The samples were electrophoresed in TAE buffer for 45 min, at 80 V and visualised under UV light.

2.5.4.2. In vitro translation

In vitro translation of total RNA was performed using a Rabbit Reticulocyte Lysate System (Promega) following the manufacturers' instructions, using a complete amino acid mixture and omitting radiolabelled amino acids. Translation reactions contained 6 μg total RNA. After the translation, protein concentration in the crude reaction mixture was determined (section 2.4.4) and DCA-*N*-MT activity was assayed as described in section 2.3.8.3 but the reaction was carried out for 1 h 30 min instead of 5 min.

2.6. Fluorescence two-dimensional differential gel electrophoresis (2-D DiGE)

2.6.1 Sample preparation

Soybean plants were grown in vermiculite as described above. After 10 d, vermiculite was washed from the roots and the plants were separated into two batches. One batch was placed in distilled water containing 100 μM DCA for 12 h and the remaining batch was placed in distilled water containing methanol only. The roots were washed with distilled water and excised. DCA-*N*-MT was partially purified from each batch of roots (approx. 150 g) using a modification of procedure 2 (section 2.4.3), with the addition of 1 mM DTT to buffers to preserve DCA-*N*-MT activity. Following the first anion exchange step, proteins were separated by gel filtration, as in step 5''. DCA-*N*-MT activity was monitored throughout the purification procedure to identify peak fractions and to compare the two treatments. Labelling and analysis of differentially expressed proteins were carried out on a service basis at the Cambridge Centre for Proteomics.

Proteins were concentrated by phenol precipitation to remove DTT which interferes with DiGE labelling. Briefly, samples were vortexed with an equal vol. of TE (100 mM Tris-HCl, pH 7.8; 1 mM EDTA)-saturated phenol and incubated on ice for 10 min. Following centrifugation at 3,000 *g* for 10 min, the upper, aqueous phase

was removed. The interface and lower phase were then extracted twice with two volumes of TE-saturated phenol. Proteins in the interface were precipitated by the addition of 5 vol. of 0.1 M ammonium acetate dissolved in methanol, followed by incubation overnight at –20 °C. Proteins were collected by centrifugation at 3,000.*g* for 10 min and the pellet washed in 80 % of 0.1 M ammonium acetate (0.1 M ammonium acetate dissolved in methanol: water; 80 : 20; v/v). Following centrifugation at 3,000 *g* for 10 min the pellet was washed in 80 % (v/v) acetone and centrifugation repeated. The supernatant was removed and the pellet dried under reduced pressure to remove remaining traces of acetone.

2.6.2 DiGE labelling

Non-saturating labeling of samples was performed using NHS esters of Cy3 or Cy5 (CyDye DiGE fluors; Amersham Biosciences). Dye (200 pmol) was added to 50 µg of sample in AUT sample buffer [7 M urea, 2 M thiourea, 2 % (w/v) ASB14, 10 mM Tris pH 8.5, 5 mM magnesium acetate] to give a typical total volume of 10-15 µl and left on ice in the dark for 30 min. The reaction was quenched by the addition of 10 nmol of L-lysine and further incubation for 10 min (Ünlü *et al.*, 1997). The Cy3-labeled and Cy5-labeled samples were then combined and prepared for isoelectric focussing (IEF) by the addition of an equal volume of sample buffer [20 mg ml^{-1} DTT, 2 % appropriate IPG buffer (Amersham Biosciences), 7 M urea, 2 M thiourea, 2 % ASB14].

2.6.3 Electrophoretic analysis

Two-dimensional electrophoresis was carried out using 13 cm pH 4-7 immobilised pH gradient (IPG) strips in conjunction with an IPGPhor (Amersham Biosciences). IPG strips were rehydrated for 12 h with rehydration buffer (2 mg ml^{-1} DTT, 1 % appropriate IPG buffer, 7 M urea, 2 M thiourea, 2 % ASB14) to which the sample was added to give a final volume of 250 ml. Focussing was carried out for a total of 41700 Vhr. Separation in the second dimension was carried out using a 12 % SDS-PAGE (Hoefer SE600). Gels were scanned with the appropriate excitation and emission wavelengths for Cy3 and Cy5 using a 2920-2D MasterImager

(Amersham Biosciences). Images were exported as TIF files. False-coloration and contrast-enhancement of scans was performed with Adobe Photoshop.

For analysis by mass spectrometry, proteins were stained with Coomassie G250 and excised manually. Proteins within the gel-excised spots were digested to peptides using trypsin on a MassPrepStation (Micromass). The resulting peptides were applied to (QTof with capLC, Micromass) in conjunction with a PepMap C18 180mm internal diameter 15 cm capillary column LC MS-MS (LC Packings). Fragmentation data was used to search the National Centre for Biotechnology Information (NCBI) *Arabidopsis* and soybean databases using the MASCOT search engine (Matrix Science). Manual sequence assignment was assisted using the peptide sequencing feature of BioLynx (Micromass).

Chapter 3

Metabolism of 3,4-dichloroaniline in soybean

3.1. Introduction

The aim of this chapter was to determine the metabolic fate of DCA in soybean. The metabolism of xenobiotics has been studied in various plant systems but since it has proved difficult to compare studies of xenobiotic metabolism due to the wide range of experimental conditions employed by different researchers, there has been a move to establish standard procedures for the study of environmental chemicals with cell suspension cultures (Ebing *et al.*, 1984; Sandermann *et al.*, 1984; Harms and Langebartels, 1986). Cell suspension cultures are frequently found to be a rich source of xenobiotic-degrading enzymes and their use can reduce the potential problem of microbial degradation of the xenobiotic. Furthermore, the ability to grow cultures in the dark ensures the absence of photodegradation. Excised leaves, as a system closer to whole plants, have also been used to study the metabolism of xenobiotics such as DCA (Gareis *et al.*, 1992). However, both cell suspension cultures and excised leaves are artificial systems and the observations obtained may not represent the actual phenomena occurring within whole plants. Therefore, DCA metabolism in whole, hydroponically-grown soybean plants was studied in this project. A similar study has been carried out by Bockers *et al.* (1994), but the present project had two additional longer-term goals to extend these observations:

1) To follow up the fate of DCA metabolites *in planta* with studies of their transport across biological membranes and to interpret this information in the context of known pathways of xenobiotic detoxification;

2) To investigate whether the mechanisms of DCA metabolism are present in other dicots, by comparison with a parallel study of DCA metabolism in *Arabidopsis thaliana* (C. Loutre, University of Durham).

3.2. Results

3.2.1. Distribution of radiolabel following [UL-^{14}C]-DCA treatment

In order to obtain an overall picture of DCA metabolism in soybean plants, 10 d-old soybean seedlings were treated with 37 kBq [UL-^{14}C]-3,4-dichloroaniline, in hydroponics. After 1 d, 3 d and 5 d, the plants were removed from the nutrient medium, rinsed thoroughly, dried and the radiolabelled compounds were visualised by phosphor-imaging to determine the distribution of radiolabelled DCA-metabolites within the plants (figure 3.1). The images showed that radiolabel accumulated mostly in the roots and in the newly-formed leaf tissue. No label could be detected in cotyledons and whilst newly-formed leaves contained ^{14}C, the signal declined as leaves expanded.

To study more precisely the distribution of DCA metabolites throughout the seedling, hydroponically-grown 10 d-old soybean seedlings were treated with 37 kBq [UL-^{14}C]-DCA in a volume of 100 ml. After 3.5 d, duplicate plants were dissected into roots, cotyledons, primary leaves, secondary leaves, and stem. DCA metabolites were then extracted from each plant sample and from the media, and quantified by LSC. After 3.5 d incubation with [UL-^{14}C]-DCA, approximately 78 % radioactivity was found in the growth medium (figure 3.2.A). Consistent with the observations obtained by phosphor-imaging, most radiolabelled metabolites within soybean plants were found in the roots and in the meristem (radioactivity expressed as dpm g^{-1} fresh weight tissue; figure 3.2.B). When expressed as a percentage of the total radioactive dose within the seedling, approximately 90 % radiolabel was present in the roots, 4-9 % in the stem and less than 1 % in the cotyledons and the primary and secondary leaves (figure 3.2.C).

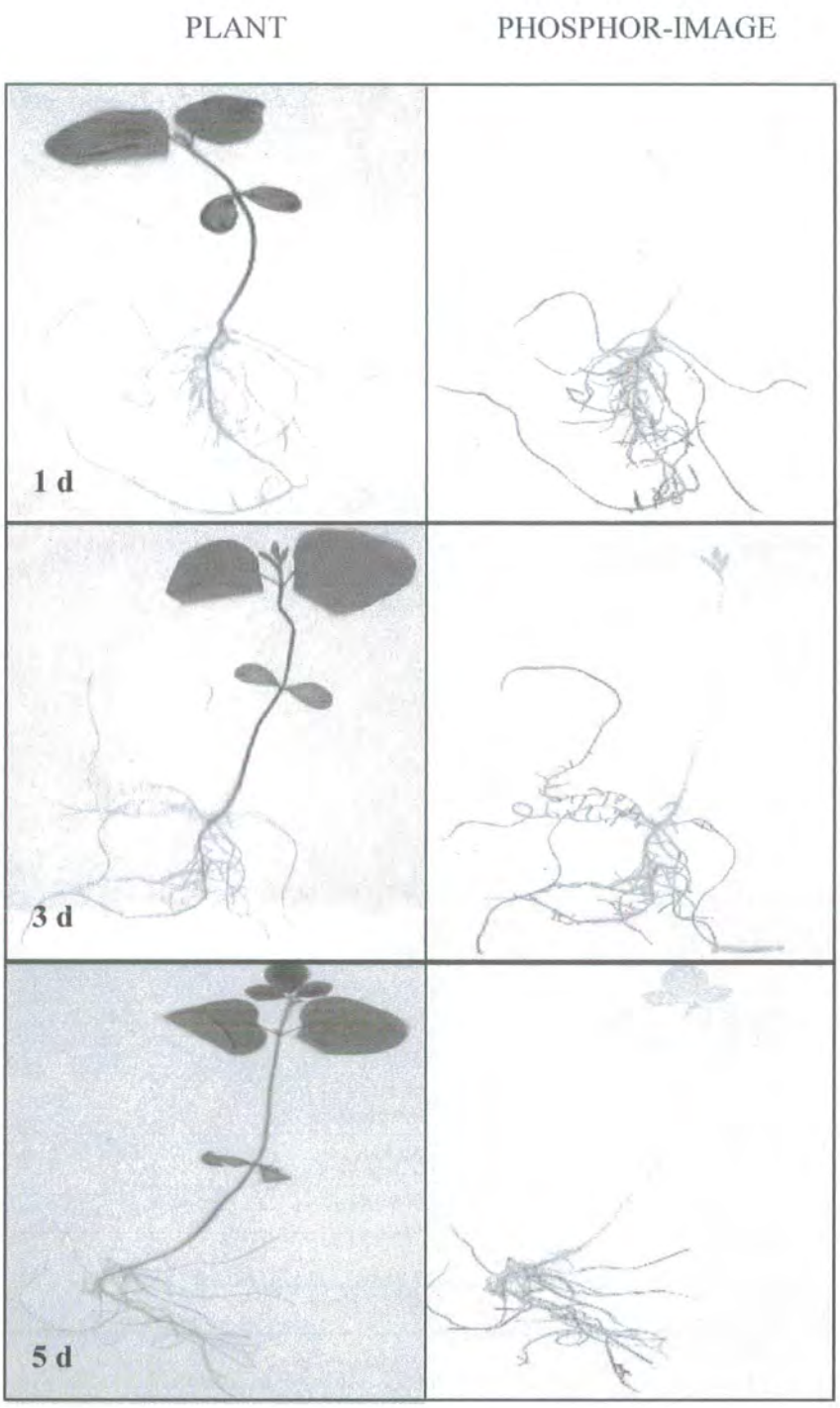


Figure 3.1: Localisation of radiolabelled compounds in soybean plants.
Soybean was grown hydroponically in the presence of [UL-^{14}C]-3,4-dichloroaniline (37 kBq, 0.43 μM). After 1-5 d, the plants were rinsed and dried. The left panels show photographs of the plants, the right panels show the corresponding phosphor-image.

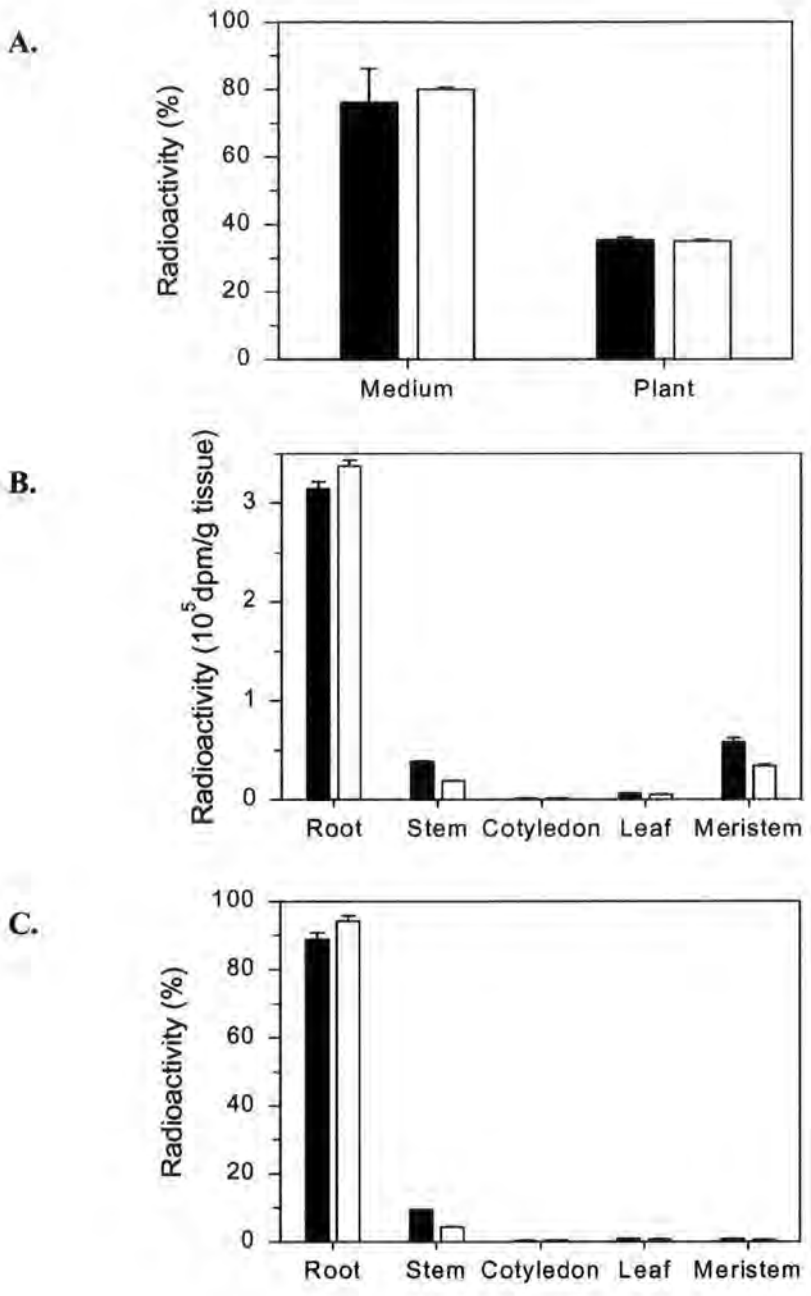


Figure 3.2: Distribution of [UL-^{14}C]-DCA metabolites between the growth medium and the soybean seedling.
Two batches of 10 d-old soybean seedlings (3 per batch) were treated hydroponically with 37 kBq [UL-^{14}C]-DCA (0.43 µM) for 3.5 days. DCA metabolites were then extracted as described in 2.3.2 and 2.3.3 and analysed by LSC. The black and white bars represent 2 independent experiments. **A.** Distribution of radioactivity between nutrient medium and plants. Figures are given as percentage of initial radioactive dose. **B and C.** Distribution of radioactivity within the plant. B, figures are given as radioactivity per gram fresh weight tissue. C, figures are given as percentage of radioactive dose taken up by the plant.

3.2.2. Identification of [UL-^{14}C]-DCA metabolites in soybean

Having determined the general distribution of radiolabel in soybean grown in the presence of [UL-^{14}C]-DCA, the identification of the metabolites was attempted. 10 d-old soybean seedlings were treated with 37 kBq [UL-^{14}C]-DCA, in 50 ml hydroponics. At different time-points (up to 48 h), DCA metabolites were extracted from the growth medium and from the roots and were analysed by TLC. Unlabelled and radiolabelled M-DCA and G-DCA conjugates were synthesised as described in section 2.1.3 and used as reference compounds.

In all chromatograms (figures 3.3, 3.5 and 3.6), the parent [UL-^{14}C]-DCA ran as three radioactive entities, with two major spots around Rf = 0.78 (DCA) and a minor spot at Rf = 0.44 (DCA-minor). As shown below (section 3.2.3.2), DCA-minor was not metabolised by soybean plants, therefore only the fate of major DCA spots was analysed further. Prior to this experiment, a relationship between M-DCA migration and its loading-dose was observed (data not shown). When more than 200 nmoles M-DCA were separated by TLC, the chemical migrated as 2 distinct spots. When the TLC plate was turned by 90 °C to allow a second dimension separation of these spots in the same solvent, the previously distinct spots migrated identically, suggesting that, when overloaded, a single compound could migrate as two different spots. To verify this, the two putative [UL-^{14}C]-DCA spots that migrated around Rf = 0.78 were scraped off and re-run in the same chromatography system. Both were found to have the same Rf value, which corroborates our initial hypothesis. Moreover, HPLC analysis of the same samples showed a single peak where reference DCA ran. Therefore, the two distinct spots corresponded to a single compound: [UL-^{14}C]-DCA. Consequently, in all subsequent analyses, these two radioactive bands were collectively quantified as parent [UL-^{14}C]-DCA.

In the plants, 7 radioactive metabolites were detected (figure 3.3). Of these, R1-R3 and R5 were identified by co-chromatography as DCA, G-DCA, DCA-minor and M-DCA, with Rf values of 0.78, 0.56, 0.41 and 0.34, respectively. R4, R6 and R7 were not identified. Bockers and co-workers (1994) suggested the presence of 6'-*O*-malonyl-*N*-(β-D-glucopyranosyl)-3,4-dicloroaniline (M-G-DCA) in soybean roots. This compound being more hydrophilic than any of the metabolites identified, R6 or R7 would be possible candidates. Therefore, root samples were separated by

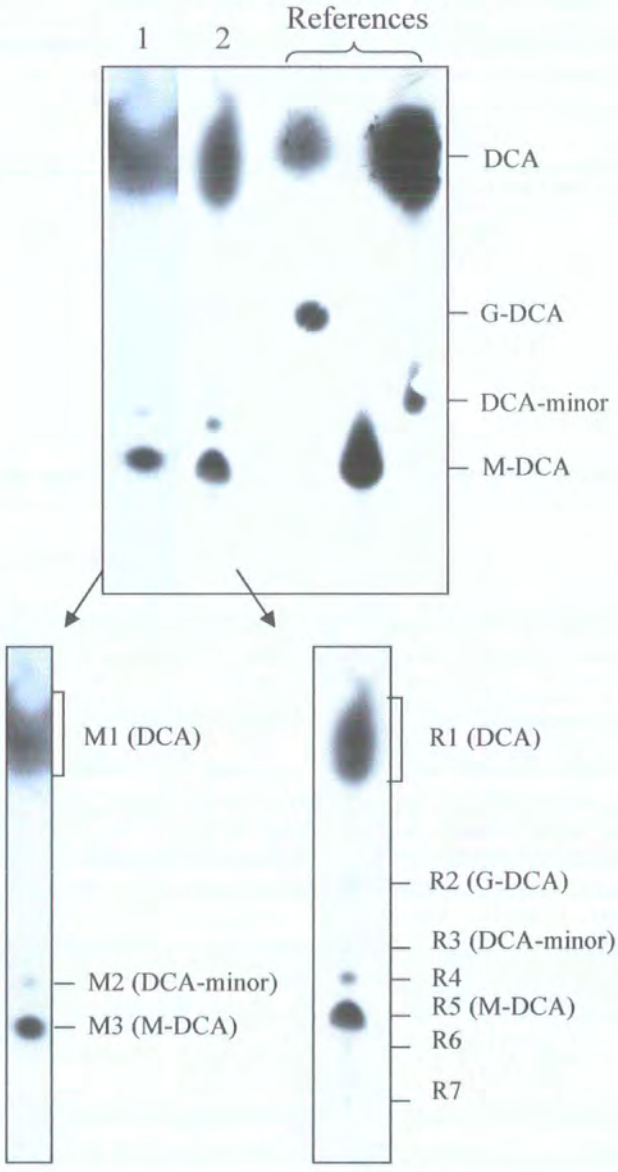


Figure 3.3: Identification of [UL-^{14}C]-DCA metabolites by TLC.

Soybean plants were treated hydroponically with 37 kBq [UL-^{14}C]-DCA for 72 h. After 4 h, radioactive compounds were extracted from the nutrient media and the roots, as described in sections 2.3.2 and 2.3.3. The samples were separated by TLC using ethyl acetate: formic acid: water (70/4/4; v/v/v) and visualised by autoradiography. Lane 1 comes from a different TLC plate. Lane 1: growth medium extract; lane 2: root extract. Radiolabelled reference compounds: G-DCA (Rf = 0.56), M-DCA (Rf = 0.34) and DCA (Rf = 0.78). Radiolabelled metabolites were detected in the growth medium (M1-M3) and in soybean roots (R1-R7).

TLC using the solvent mix Bockers and co-workers utilised (chloroform: methanol: water, 65/25/4, v/v/v) to compare directly the Rf values of the different compounds. Surprisingly, the Rf values obtained for standard compounds were different from those published by Bockers *et al.* (data not shown), rendering a direct comparison of DCA metabolites impossible. As a consequence, an alternative method was tested: to determine whether any metabolites corresponded to M-G-DCA, the detection of *O*-malonylated compounds was attempted. Root extracts were treated with 0.2 M Tris to cleave the malonyl group from the G-DCA (Tiller *et al.*, 1994) and re-analysed by HPLC. Unfortunately, the compounds remained unidentified (data not shown). Therefore, the presence or absence of M-G-DCA in our samples could not be determined.

In the growth medium, M1-M3 were identified by co-chromatography as DCA, DCA-minor and M-DCA, respectively (figures 3.3 and 3.5). Therefore, apart from the parent [UL-^{14}C]-DCA, a single metabolite was found in the nutrient medium, which corresponded to the malonyl conjugate M-DCA. This observation was confirmed by HPLC (data not shown) and was consistent with published data (Winkler and Sandermann, 1989).

3.2.3. Time-course study of DCA metabolism in soybean plants

3.2.3.1. Partition of radiolabelled DCA-metabolites

To provide a more detailed understanding of DCA metabolism in soybean plants, a time course experiment was carried out in which replicate seedlings were grown in the presence of radiolabelled DCA. At various time-points over a 48 h period, metabolites were extracted from the plants and growth media. In the seedlings, soluble metabolites were extracted in methanol and metabolites from the growth media were extracted with ethyl acetate (section 2.3.3). Non-extractable radioactivity in the plant material was quantified after alkaline hydrolysis (section 2.3.2) and LSC. As shown in figure 3.4, the amount of radioactivity in the media decreased rapidly with a simultaneous increase in the plants. At 30 min, 15-20 % radioactivity was found in the plants. This increased to approximately 30 % at 2 h and reached a plateau after 4 h. In the plants, the level of radioactivity found in the soluble fraction and the bound residues augmented in a similar fashion, with a slight

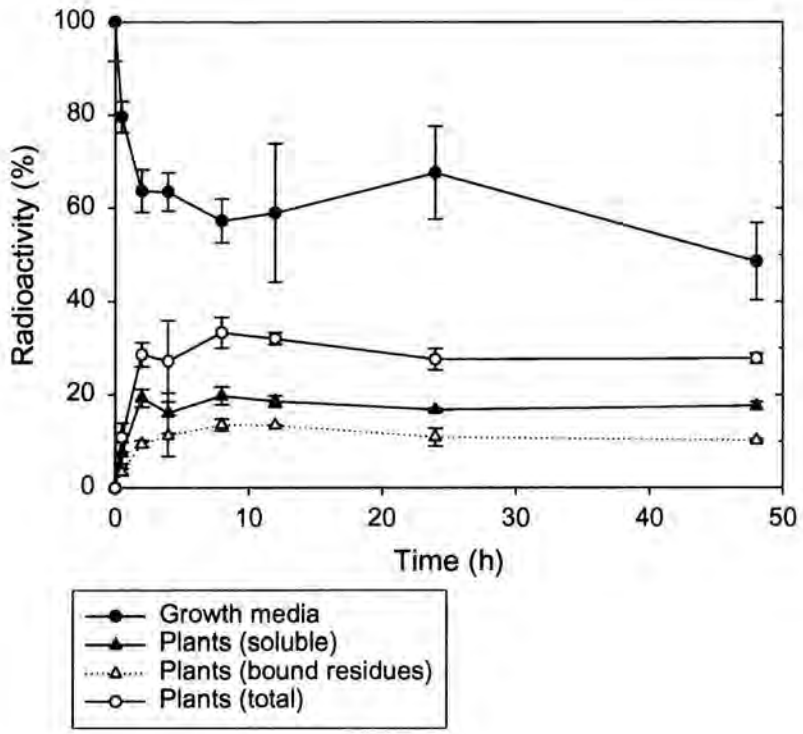


Figure 3.4: Time-course of [UL-^{14}C]-DCA uptake and distribution in soybean. Soybean plants grown hydroponically were treated with 37 kBq [UL-^{14}C]-DCA (final concentration: 0.86 μM) for 48 h. At various time-points, the radioactive metabolites were extracted from the plants and the growth media (as described in sections 2.3.2 and 2.3.3) and quantified by LSC. Representative of four experiments.

preferential partition into the soluble fraction: approximately 20 % of the applied radioactivity was found in the soluble extract compared to 12 % in the bound residues. The total radioactivity recovered decreased with time. At 48 h, only 80 % radioactivity was recovered, probably due to the continuous aeration of the growth media during the experiment, which resulted in volatilisation of DCA.

3.2.3.2. Variation of radiolabelled DCA-metabolites with time

The quantification of radiolabel in the plants and growth media gave an indication of metabolite partition during DCA metabolism but did not supply any information as to the identity of the metabolites. Although the main metabolites detected during [UL-^{14}C]-DCA metabolism were identified in the previous section, the transformation of DCA to its conjugates is a dynamic process that cannot be fully understood by the analysis of a single time-point. Therefore, a time-course study of [UL-^{14}C]-DCA metabolism in soybean seedlings was carried out, in which the qualitative analysis of radiolabelled compounds within soybean plants and the growth media was performed, using TLC. As stated above (section 3.2.1.1), 90 % of the radioactivity within a seedling grown in the presence of [UL-^{14}C]-DCA was located within the roots. The quantity of radioactivity within the shoots being very low, it was technically impossible to perform a qualitative study of [UL-^{14}C]-DCA metabolites present in this organ using TLC. Consequently, TLC analyses were performed only with samples from roots and growth media. TLC plates were then analysed using a phosphor-imager (with the assistance of M. Brazier, University of Durham) to quantify the radioactivity of each DCA metabolite at each time-point.

In the growth media, 3 metabolites were detected and identified as the parent [UL-^{14}C]-DCA, its malonyl conjugate and DCA-minor (figures 3.3 and 3.5). A visual inspection of the TLC plate, followed by the analysis of the radioactive content of each spot by phosphor-imager (table 3.1) demonstrated that: (1) DCA-minor represented approximately 6 % of the total radioactivity per lane at any time-point, suggesting that it was not metabolised during the time-course experiment. (2) As the parent [UL-^{14}C]-DCA disappeared from the growth medium, M-DCA appeared. Thus, at 30 min, the proportion of [UL-^{14}C]-DCA and its malonyl conjugate were 93.5 % and 6.5 %, respectively. These figures changed rapidly to

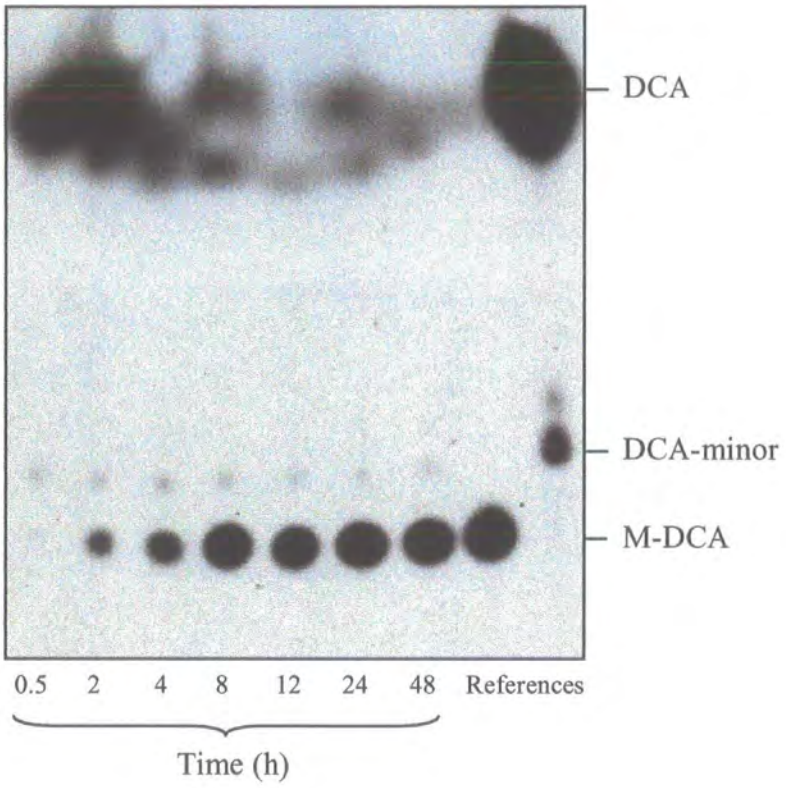
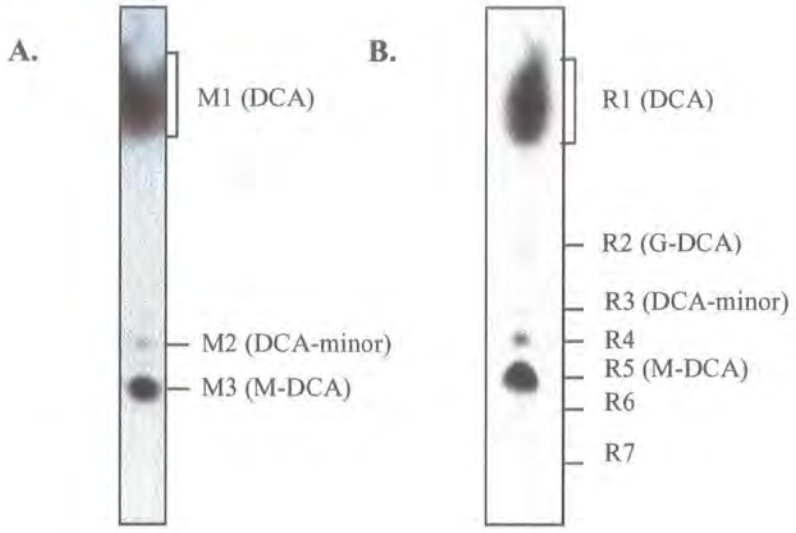


Figure 3.5: Time-course analysis of [UL-^{14}C]-DCA metabolites in the growth media.

Soybean plants were grown hydroponically in the presence of [UL-^{14}C]-DCA for 48 h. At different time-points between 30 min and 48 h, radioactive compounds were extracted from the roots, as described in section 2.3.3, separated by TLC using ethyl acetate: formic acid: water (70: 4: 4; v: v: v) and visualised by autoradiography. Radiolabelled reference compounds: M-DCA (Rf = 0.34) and DCA (Rf = 0.78).



	Nutrient medium		Soybean root		
Time (h)	DCA	M-DCA	DCA	R4	M-DCA
0.5	93.5	6.5	70.7	3.6	25.7
2	77.0	23.0	63.3	5.8	30.9
4	52.3	47.7	68.5	6.2	25.3
8	26.8	73.2	83.3	7.2	9.6
12	19.3	80.7	83.7	6.8	9.4
24	18.8	81.2	79.1	3.5	17.4
48	18.1	81.8	72.3	3.2	24.5

Table 3.1: Variation of DCA metabolites with time, in soybean.

Soybean plants were grown hydroponically in Hoagland's nutrient medium, with 37 kBq [UL-^{14}C]-DCA, for 48 h. At different time-points between 30 min and 48 h, radioactive compounds were extracted from the nutrient media and soybean roots and were separated by TLC using ethyl acetate: formic acid: water (70/4/4; v/v/v). TLC plates were then analysed using a phosphor-imager to quantify the radiolabelled species. Radioactivity in each spot given as a percentage of the total radioactivity per track. A: Radiolabelled compounds extracted from the growth media. B: Radiolabelled compounds extracted from soybean roots. Only spots M1-M3, R1 and R3-R5 were detected by phosphor-imaging. M2 and R3 (6 % radioactivity) were not included in the table because they did not vary with time.

19.3 % and 80.7 %, respectively, at 12 h and stabilised afterwards. These results suggested that [UL-^{14}C]-DCA was taken up by soybean roots, conjugated to a malonyl residue and released into the medium within 30 min.

In soybean roots, 7 metabolites were detected by autoradiography (figure 3.3). Figure 3.6 shows the autoradiograph of the TLC plate on which root extracts were analysed, during the time-course experiment. Apart from R2 (G-DCA), which was present from 30 min to 4 h and disappeared afterwards, other metabolites were detected at all time-points, but in variable quantities. As observed in the growth media, the amount of DCA-minor did not vary during the time-course experiment (figure 3.5) and here, was found to represent 5.5 % of the total radioactivity present in each lane. This is consistent with the 6 % detected in the medium, suggesting that DCA-minor was not metabolised within soybean roots either. Further analysis of root DCA metabolites by phosphor-imaging thus excluded DCA-minor. Phosphor-imager analysis of the same TLC plate only showed the presence of R1 (DCA), R4 (unknown) and R5 (M-DCA) (data not shown), suggesting that the plate might not have been exposed long enough to detect the presence of lower amounts of radiolabel. The variation of R1, R4 and R5 with time is illustrated in table 3.1. The level of all three compounds varied with time, with no obvious trend. Most radiolabelled product present in the roots consisted of the parent [UL-^{14}C]-DCA (63.3-83.7 %). The second greatest metabolite was M-DCA (9.4-30.9 %) and the unidentified R4 was minor (3.2-7.2 %). Due to the low radioactive level in bound residues, combined with the high salt content of the samples, their qualitative analysis using TLC was technically difficult, hence not achieved.

3.2.4. Comparison with DCA metabolism in *Arabidopsis* root cultures

In a parallel study, *Arabidopsis thaliana* root cultures were treated with 37 kBq [UL-^{14}C]-DCA (conducted by C. Loutre, University of Durham, using protocols developed for soybean). The data are summarised here for comparison with those from soybean. At various time-points over a 48 h period, metabolites were extracted from the roots and growth media as described for soybean (section 3.2.3.1) and radioactivity quantified by LSC. Unlike soybean plants, which accumulated approximately 30 % radioactivity at 48 h (figure 3.4), nearly 60 % radioactivity was present in *Arabidopsis* root cultures at the same time-point (figure

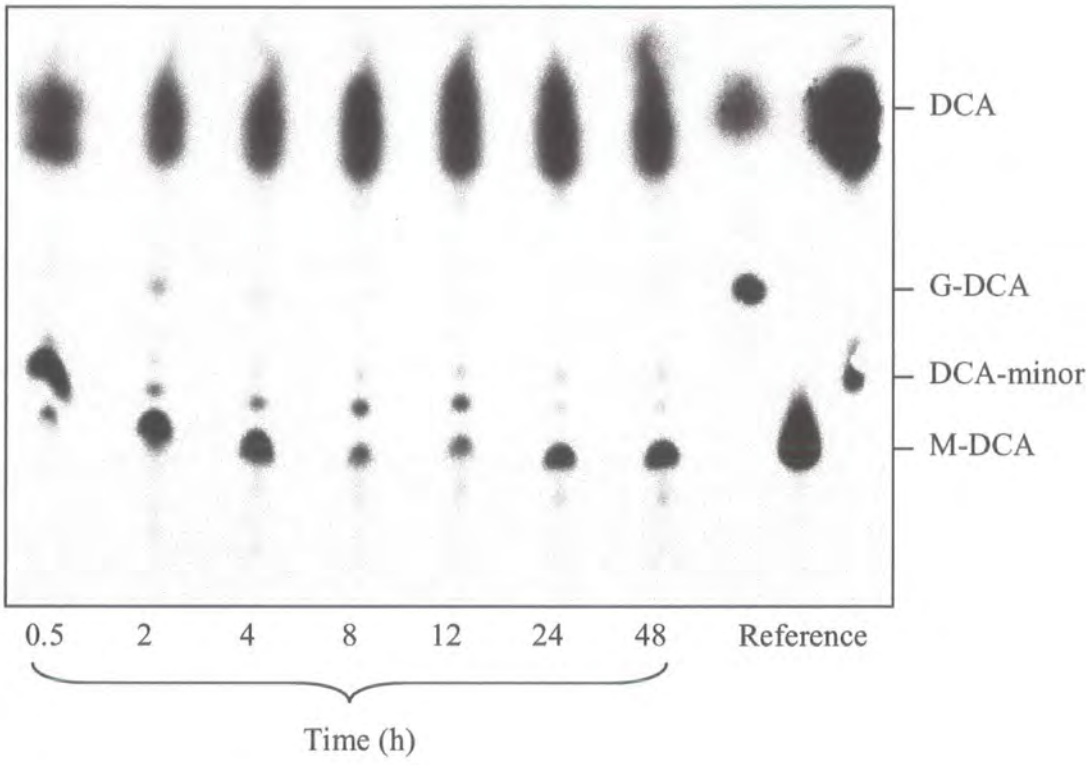


Figure 3.6: Time-course analysis of [UL-^{14}C]-DCA metabolites in soybean roots.

Soybean plants were grown hydroponically with [UL-^{14}C]-DCA, for 48 h. At different time-points between 30 min and 48 h, radioactive compounds were extracted from the nutrient media, as described in section 2.3.2, separated by TLC using ethyl acetate: formic acid: water (70: 4: 4; v: v: v) and visualised by autoradiography. Radiolabelled reference compounds: G-DCA (Rf = 0.56), M-DCA (Rf = 0.34) and DCA (Rf = 0.78).

3.7). Another difference in DCA metabolism in *Arabidopsis* and soybean was that whilst up to 12 % radioactivity was found in the bound residues of the latter, less than 3 % was present in *Arabidopsis*.

In order to identify the radiolabelled metabolites present in the nutrient medium and in the plant, samples were analysed by TLC using the following solvent mixture: chloroform/methanol/water; 60/35/8; v/v/v. Figure 3.8 is an autoradiograph of the TLC plate. At 48 h, two main metabolites were detected in the growth medium in addition to the parent DCA (figure 3.8). The most abundant, A3, was identified as the glucosyl conjugate of DCA by co-chromatography with enzymatically synthesised G-DCA. A2, which migrated close to G-DCA, remained unidentified but is probably also a glucoside (C. Loutre, pers. comm.). Phosphor-image analysis of the metabolites during a time-course experiment (table 3.2) demonstrated that, from 4 h to 48 h: (1) the proportion of DCA decreased from 29 % to 10.3 % while the amount of A2 increased simultaneously (up to 24.3 %); (2) the major metabolite present was the glucosyl-conjugate of DCA, the amount of which was virtually stable over the course of the experiment (average of 65 %); (3) very little M-DCA was detected (less than 5 %). These observations suggested that DCA was taken up by *Arabidopsis*, metabolised to predominantly to G-DCA but also to A2 and M-DCA and released into the external medium.

In the roots, six different metabolites were detected after 48 h. Of these, A1, A3 and A4 were identified as the parent DCA, G-DCA and M-DCA, respectively (with Rf values of 0.76, 0.49 and 0.44 respectively). The rest remained unidentified. The relative proportions of metabolites A1-A6 with time was analysed by phosphor-imaging. The results are summarised in table 3.2. In roots, the amount of DCA and A2 did not vary greatly from 4 h to 48 h. Conversely, while G-DCA decreased from 70.6 % to 28.3 % of the total radioactivity, the proportion of M-DCA, A5 and A6 increased (up to 12-fold higher for A6) suggesting that although G-DCA is exported from the roots, some of it might be converted to M-DCA, A5 and A6. In agreement with this, the transformation of G-DCA into M-DCA has been reported (Bockers *et al.*, 1994).

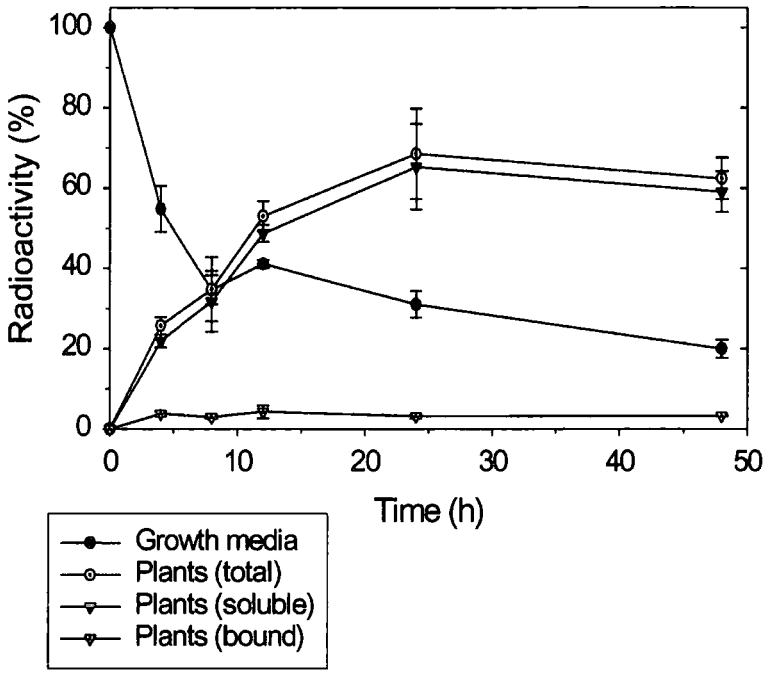


Figure 3.7: Time-course of [UL-^{14}C]-DCA uptake and distribution of label in *Arabidopsis thaliana*.

Data from C. Loutre (University of Durham), presented for comparison with soybean. *Arabidopsis* root cultures were treated with 37 kBq [UL-^{14}C]-DCA (final concentration: 0.86 µM) for 48 h. At various time-points, the radioactive metabolites were extracted from the plants and the growth media (as described in sections 2.3.2 and 2.3.3) and quantified by LSC.

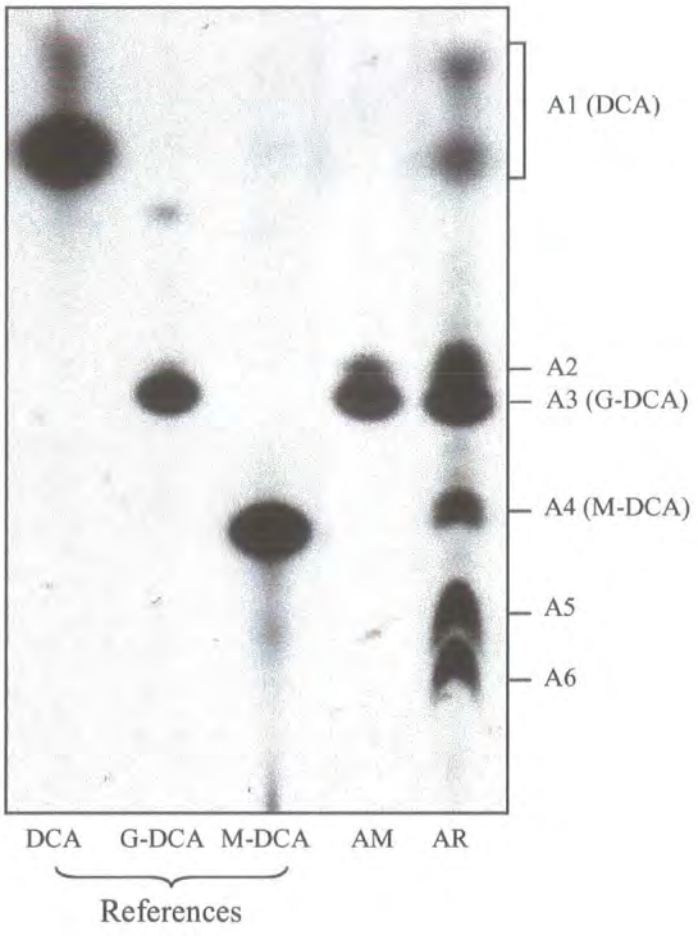
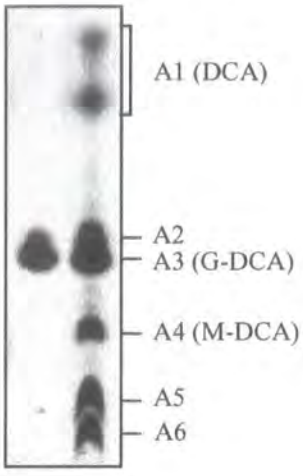


Figure 3.8: Analysis of [UL-^{14}C]-DCA metabolites in *Arabidopsis thaliana*.
Arabidopsis root cultures were treated with 37 kBq [UL-^{14}C]-DCA (final concentration: 0.86 µM), for 48 h. Radioactive compounds were extracted from the nutrient medium (AM) and the roots (AR), as described in section 2.3.2, separated by TLC using chloroform: methanol: water (60/35/8; v/v/v) and visualised by autoradiography. Radiolabelled reference compounds: DCA (Rf = 0.76), G-DCA (Rf = 0.49) and M-DCA (Rf = 0.44). A1-A6: radiolabelled DCA-metabolites detected (*Arabidopsis* samples were provided by C. Loutre, TLC analysis was performed by S. Lao).



Time (h)		DCA	A2	G-DCA	M-DCA	A5	A6
4	Medium	29.1	3.5	67.4	ND		
8		20.1	15.1	64.7	ND		
12		24.9	13.1	57.3	4.7		
24		7.3	19.0	72.3	1.4		
48		10.3	24.3	63.1	2.3		
4	Roots	10.2	12.2	70.6	1.6	3.7	1.6
8		13.7	15.7	60.1	2.0	5.9	2.6
12		17.9	13.0	56.5	2.2	7.1	3.3
24		15.3	13.9	45.3	4.4	11.7	9.5
48		12.1	9.1	28.3	7.1	24.2	19.2

Table 3.2: Variation of DCA metabolites with time, in *Arabidopsis thaliana*.
Arabidopsis root cultures were grown with 37 kBq [UL-^{14}C]-DCA for 48 h. At different time-points between 4 h and 48 h, radioactive compounds were extracted from the nutrient media and the roots and were separated by TLC using chloroform: methanol: water (60/35/8; v/v/v). TLC plates were then analysed by phosphor-imager to quantify the radiolabelled species. Radioactivity in each spot given as a percentage of the total radioactivity per track.

3.2.5. Transferase activities

In order to investigate the mechanisms underlying the differences in DCA conjugation in soybean and *Arabidopsis*, the activities of DCA-*N*-MT and 3,4-dichloroaniline-*N*-glucosyltransferase (DCA-*N*-GT) were determined in crude root extracts from the two species (table 3.3). In both cases, product formation was monitored by quantifying the amount of radiolabelled M-DCA or G-DCA formed by partitioning into ethyl acetate (sections 2.3.8 and 2.3.9).

In soybean roots, DCA-*N*-MT activity was high (12,000 pkat g^{-1}), whereas DCA-*N*-GT was just detectable (0.5 pkat g^{-1}; table 3.3). In *Arabidopsis* root cultures, both DCA-*N*-GT and DCA-*N*-MT activities were readily detectable but DCA-*N*-GT activity was much higher. No DCA-*N*-MT or DCA-*N*-GT activity could be determined in the media immersing either the soybean or *Arabidopsis* roots, confirming that the synthesis of the respective conjugates occurred within the plant tissues. In soybean suspension-cultured cells, DCA-*N*-MT activity is nearly 20-fold higher than that in the roots, which is consistent with previously published data suggesting that cell suspension cultures catabolise xenobiotics in a qualitatively similar way to whole plants, although much more effectively (Höhl and Barz, 1995; Schmidt *et al.*, 1995). Thus, the relative activities of DCA-*N*-GT and DCA-*N*-MT in soybean and *Arabidopsis* agree with the metabolic fate data presented above, in accordance with the proposition of Schmidt and co-workers (1995) that the metabolism of xenobiotics correlates strongly with the species- and organ-specific activities of transferase enzymes.

3.2.6. Transport studies

3.2.6.1. Cross-feeding

In sections 3.2.3 and 3.2.4, it was demonstrated that DCA is exported from roots in a species-specific manner, predominantly as M-DCA in soybean and G-DCA in *Arabidopsis*. Consequently, a cross-feeding experiment (performed jointly with C. Loutre, University of Durham) was conducted to determine:

Plant source	DCA-*N*-MT activity (pkat g^{-1})	DCA-*N*-GT activity (pkat g^{-1})
10 d-old soybean roots	12,000.0	0.5
10 d-old soybean shoots	300.0	Negligible
Suspension-cultured cells	230,000.0	6.0
14 d-old *Arabidopsis* root culture	52.0	6,000.0

Table 3.3: DCA-*N*-MT and DCA-*N*-GT activities in soybean and Arabidopsis tissues.

DCA-*N*-MT and DCA-*N*-GT activities were determined as described in sections 2.3.8.2 and 2.3.9, respectively. DCA-*N*-MT activity was measured in crude protein extracts (section 2.4.1). DCA-*N*-GT activity was measured in desalted ammonium sulphate precipitates, as described in section 2.4.2, step 2, except that the desalting buffer consisted of 0.2 M Tris-HCl, pH = 8.0, 2 mM DTT.

(1) whether M-DCA could be re-absorbed by soybean plants;

(2) whether M-DCA could be taken up by a plant species in which it is not abundantly synthesised, i.e. *Arabidopsis thaliana*;

(3) whether soybean plants were able to take up DCA metabolites originating from *A. thaliana.*

Soybean seedlings and *Arabidopsis* root cultures were grown in the presence of 37 kBq [UL-^{14}C]-DCA. After 48 h, the metabolites released into the medium by each plant species were extracted with ethyl acetate, concentrated and quantified by LSC. After 48 h feeding, the radiolabelled metabolites present in the soybean nutrient medium consisted of approximately 80 % M-DCA, the rest being the parent DCA (table 3.1). The *Arabidopsis* medium contained approximately 63 % G-DCA, 10 % DCA, 24 % A2, 2 % M-DCA, the rest being unidentified compounds (section 2.3.4). Untreated plants from both species were then incubated for 24 h in 20 ml fresh medium containing 6.67 kBq radiolabelled metabolites from each species. Plants and media were analysed by LSC to determine the partition of ^{14}C between the growth medium and plant material in the two species.

Soybean seedlings took up approximately equal amounts of radiolabelled compounds whether they were derived from soybean or *Arabidopsis* growth medium. In each case, 12 % of the supplied radioactivity was recovered in the soluble fraction while 5 % became incorporated into bound residues, regardless of source of the metabolites (figure 3.9). At the beginning of the cross-feeding experiment, the metabolites present were: 80 % M-DCA and 20 % DCA. Assuming that only the parent DCA was metabolised, 94 % of the total radioactivity would be found in the growth medium and 6 % would be in the plants, which is not what was obtained in figure 3.9.A. Consequently, this suggested that a small proportion of the DCA-metabolites could be taken up by soybean. However, having no evidence that soybean behaves in a similar fashion when treated with DCA + M-DCA and with DCA only, no definitive explanation could be given.

Arabidopsis root cultures showed a slight preference for the uptake of *Arabidopsis* DCA-metabolites, with 17 % of the radioactivity recovered from the roots, compared with only 6 % when the medium contained soybean DCA metabolites (figure 3.9.B). Almost no bound residues were formed in *Arabidopsis* ($\geq$ 3 %). Assuming that only the 10 % DCA present at the beginning of the cross-

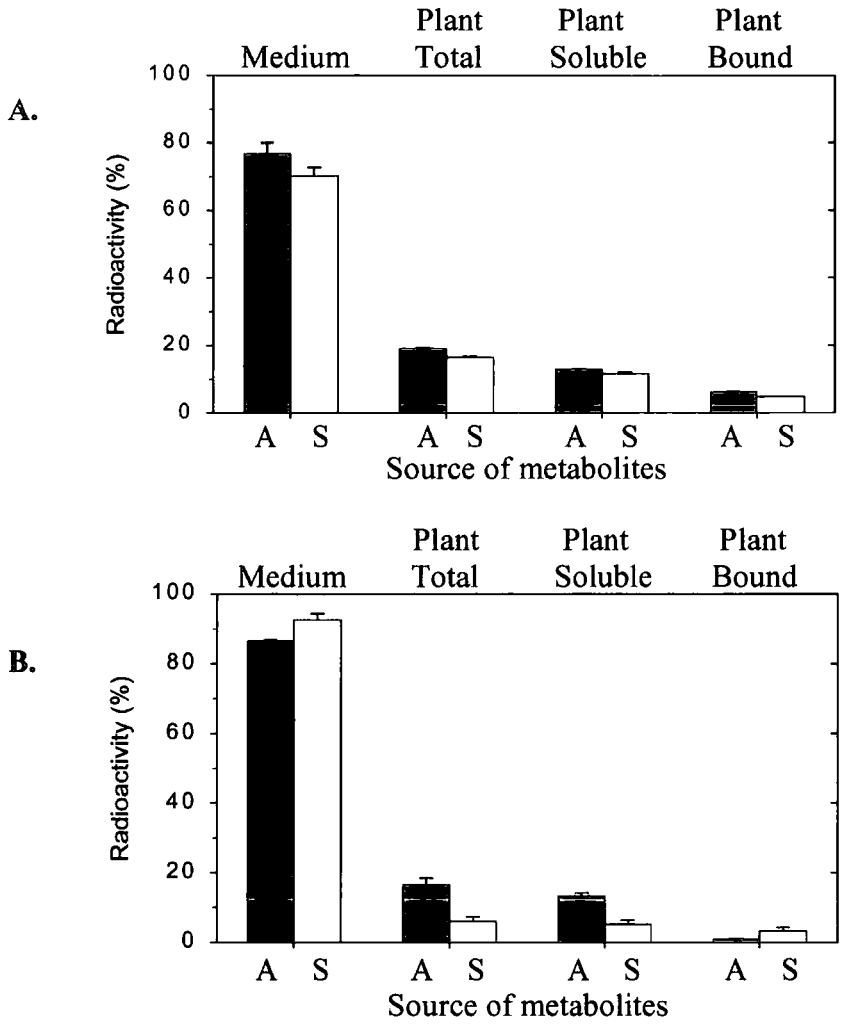


Figure 3.9: Partition of radiolabelled compounds between *Arabidopsis*, soybean and nutrient medium, when grown with *Arabidopsis* and soybean DCA-metabolites.

Soybean seedlings and *Arabidopsis* root cultures were grown in the presence of 37 kBq [UL-^{14}C]-DCA as described in section 2.3.11. After 48 h, the metabolites released into the medium by each plant species were extracted, concentrated and quantified by LSC. Untreated plants were then incubated for 24 h in 20 ml fresh medium containing 6.67 kBq radiolabelled metabolites from each species. Plants and media were then analysed by LSC to determine the partition of radiolabel between the growth medium and the two plant species. Results are given as percentage of initial DCA metabolite dose. A: Radioactive content of soybean roots and medium after feeding with *Arabidopsis* metabolites (black bars) and soybean metabolites (open bars); B: Radioactive content of *Arabidopsis* roots and medium after feeding with *Arabidopsis* metabolites (black bars) and soybean metabolites (open bars).

feeding were metabolised, 93 % of the total radioactivity would be found in the growth medium and 6.5 % would be in the plants. This is not what was obtained when *Arabidopsis* was grown with its own metabolites but resembles the results obtained when soybean metabolites were used (figure 3.9.B), suggesting the possible re-uptake of *Arabidopsis* DCA-metabolites by *Arabidopsis* root cultures. Due to the low radioactive content of the extracts at the end of this experiment, analyses by TLC were impossible. They would have been very informative and would have helped to clarify whether M-DCA and G-DCA were metabolised further.

3.2.6.2. Effect of transport inhibitors on the efflux of M-DCA

In order to investigate the mechanism(s) whereby malonyl-conjugates of DCA are released into the nutrient medium, the effect of transport inhibitors on M-DCA efflux was studied. Hydroponically-grown soybean seedlings were treated with 37 kBq [UL-^{14}C]-DCA. After 30 min incubation, the seedlings were rinsed thoroughly with distilled water and transferred to fresh media containing various inhibitors. After 2 h efflux of DCA metabolites, radioactivity in the nutrient medium was measured using LSC.

Figure 3.10 summarises the results of this study. Sodium azide, an inhibitor of F_1/F_0-type ATPases (present in mitochondria and thylakoids), reduced the efflux by 10 % compared to the control. This demonstrates a requirement for ATP, since azide treatment partially depletes the ATP pool in roots. In order to determine whether primary pumps were involved, three ATPase inhibitors were tested. At the concentrations used in this study, nifedipine and quinidine, inhibitors of P-glycoprotein-type ABC-transporters, did not affect the efflux of radiolabelled metabolites. Furthermore, sodium orthovanadate, an ABC-transporter and P-type ATPase inhibitor did not inhibit efflux, suggesting that a primary pump was not involved. Two anion-transporter inhibitors were utilised, namely probenecid and 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS). The latter, known to be a Cl^- channel inhibitor, had a minor effect (less than 10 % decrease) on the efflux of radiolabelled compounds from soybean roots, whereas probenecid, which inhibits organic anion transport, diminished the efflux by approximately 25 %. Treatment with butyric acid at pH 5.6 also decreased M-DCA efflux by approximately 30 %. Since butyric acid decreases the pH of the cytoplasm (Ryan and Walker, 1994),

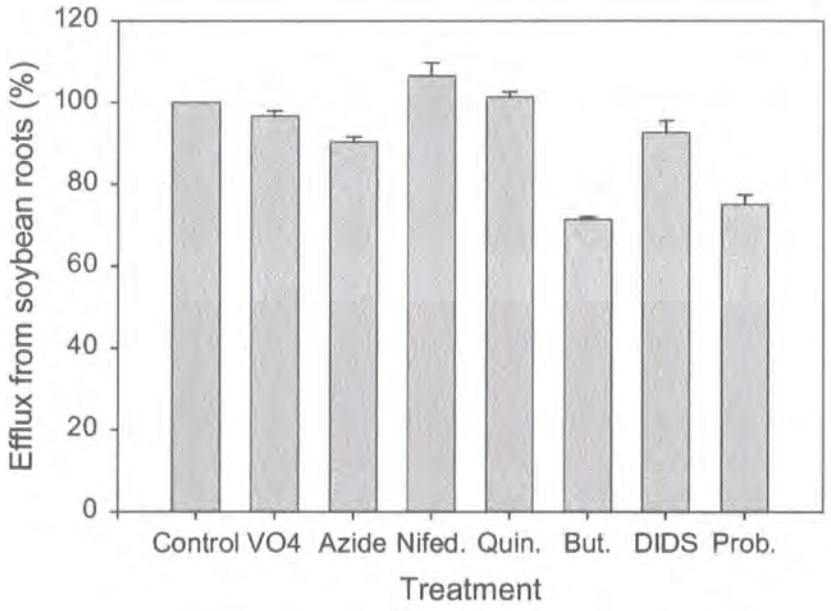


Figure 3.10: Effect of various inhibitors on M-DCA efflux from soybean roots. 12 d-old soybean seedlings were grown in a flask containing 50 ml Hoagland's (pH = 6.0) fresh medium and 37 kBq [UL-^{14}C]-DCA (3 plants/flask). After 30 min incubation at room temperature, to allow the uptake of [UL-^{14}C]-DCA by the plants, the roots were thoroughly rinsed with distilled water, transferred to fresh media containing various inhibitors and left for 2 h. The amount of radioactivity present in the media at the beginning of the experiment, after 30 min and after 2 h incubation with inhibitors, was quantified by LSC (section 2.3.4). The following inhibitors were used: 1 mM sodium orthovanadate (VO_4), 1 mM sodium azide, 50 µM nifedipine (Nifed.), 100 µM quinidine (Quin.), 10 mM butyric acid (But.), 1 mM 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) and 1 mM probenecid (Prob.). Control: water. Results given as the percentage of efflux in each treatment compared to that in the control.

transport systems driven by pH-gradients would be inhibited. The fact that azide affected M-DCA efflux and that both butyric acid and probenecid inhibited 25-30 % of radiolabel efflux suggested that a secondary transport system, probably an organic anion-carrier, that is energised by the proton motive force, was involved in the efflux of M-DCA from soybean roots. In view of this, it is perhaps surprising that no effect was seen with vanadate, which would be expected to inhibit the plasma membrane H^+-ATPase and hence reduce the trans-plasma membrane proton gradient.

3.3. Discussion

The aim of this chapter was to determine the metabolic fate of DCA in intact soybean plants and to carry out preliminary transport studies. To do so, hydroponically-grown soybean plants were treated with [UL-^{14}C]-DCA and the identity and distribution of [UL-^{14}C]-DCA metabolites studied (figures 3.2 and 3.3). The results obtained here differed quantitatively and qualitatively from previously published data. After 48 h treatment, Bockers *et al.* (1994) reported 58.8 % radioactivity in the medium and 37.3 % in the soybean plants (*Glycine max* L. var. Harosoy 63); our results were lower (48.6 % and 27.8 %, respectively), which may be due to the fact that the recovery of radioactivity in the experiment reported here was only 80 %. This loss of radiolabel may be due the volatilisation of DCA, either because of the continuous aeration of the medium or because of plant transpiration. The distribution of radioactivity translocated to the plants and the identity of the metabolites were also different. In the present study, after 3.5 d treatment with radiolabelled DCA (figure 3.2.C), about 90 % radioactivity present within the plant was located in the roots. Very little radiolabel was translocated to the shoots and most of this was located in the newly-formed leaves (figures 3.1 and 3.2.C). Bearing this in mind and the fact that the activity of neither DCA-*N*-MT nor DCA-*N*-GT is high within soybean shoots (table 3.3), it can be hypothesised that the translocation of DCA-metabolites to the shoots is not an important detoxification mechanism in soybean var. Chapman. Surprisingly, Bockers and co-workers (1994) obtained very different results. They grew soybean plants hydroponically and treated them with radiolabelled DCA. After 48 h and 120 h, the plants and media were analysed. At 48 h, they found only 50 % radioactivity in the roots and 27 % in the shoots. After 5 d,

the radiolabel present in the roots had decreased to 25.7 % and 39 % had translocated to the shoots. Moreover, the proportion of bound residues reported (23.5 % and 35.2 % after 48 h and 5 d, respectively) was much higher than that obtained here (approximately 10 %, figure 3.4). This suggests that the translocation of DCA metabolites to the shoots and their partial integration into bound residues is an integral part of DCA detoxification mechanisms in soybean (var. Harosoy 63). Consequently, it appears that even within one plant species, DCA metabolism can take very different routes.

Differences in the identity of DCA metabolites were also observed. Generally, Bockers and co-workers (1994) detected high proportions of glucosyl-conjugates in every organ studied whereas in this study, M-DCA was the major conjugate synthesised. According to their results, G-DCA and M-G-DCA represented 27.3 % and 10.5 % of the radioactivity in their plants, but the levels of these conjugates were very low if not absent in our system. Schmidt and co-workers (1995) have reported a strong correlation between the distribution and identity of DCA metabolites and the occurrence of the respective transferase enzymes. Accordingly, the absence of these glucosyl-conjugates could be explained by the very low DCA-*N*-GT activity in the roots of the soybean (var. Chapman) utilised for the current experiments (table 3.3). Consistent with the fact that M-DCA was the major metabolite found in both the nutrient medium and soybean roots during [UL-^{14}C]-DCA metabolism (table 1), DCA-*N*-MT activity was highest in the roots (table 3.3), suggesting the importance of DCA-*N*-MT in the detoxification of DCA by soybean (var. Chapman).

After DCA-treatment, two major metabolites were detected within soybean roots and were identified as the parent DCA and its malonyl-conjugate (table 3.1). Surprisingly, the quantity of these metabolites did not vary much from 30 min to 48 h, suggesting a steady-state was reached between import, conjugation and export. At this stage, DCA and M-DCA represented approximately 70 % and 20 % of radioactivity in roots, respectively. As for the bound residues, their level reached a plateau after 8 h, which was consistent with previously published data (Schmidt, 1999).

Many conjugates of xenobiotics and natural products are stored in the vacuole or incorporated, possibly via a multi-step route, into the cell wall. For example, malonyl-conjugates of natural products, such as the ethylene precursor 1-

aminocyclopropane-1-carboxylic acid, the isoflavone phytoalexin medicarpin, or degradation metabolites of chlorophyll, are known to be compartmentalised within the vacuole (Yang and Hoffman, 1984; Barz and Mackenbrock, 1994; Hörtensteiner, 1998). Other non-malonylated conjugates of xenobiotics are believed to be translocated either into the vacuole (soluble conjugate) or into the apoplast, and bound to pectin, hemicellulose or lignin intermediates (insoluble conjugates, also known as bound residues) (Sandermann *et al.*, 1997). Here, it was shown that the malonylated conjugates of DCA were predominantly transported out of the root cell and only a small portion was present within the root cell (table 3.1). The export of M-DCA from the cell was also observed in studies utilising suspension-cultured cells of soybean and carrot (Winkler and Sandermann, 1989; Schmidt *et al.*, 1994) but is in contrast with the general dogma that xenobiotics are compartmentalised either within the vacuole or the apoplast. However, the intracellular localisation of M-DCA was not studied and therefore, its partial transport into the vacuole cannot be excluded.

The uptake and transport of compounds by plants depends on the lipophilicity (log K_{ow}) and the acid strength (pKa) of the particular chemicals. A highly lipophilic molecule partitions more readily into biological membranes and the higher its acid strength, the less likely the molecule will be ionised (it is more difficult for an ionised molecule to partition through biological membranes). DCA has a pKa of about 4, which means that as a weak base, it is not greatly protonated at pH values above or equal to 6 and thus can be considered as unionised. With a log K_{ow} = 2.69, DCA has a medium lipophilicity and thus would partition into membranes easily and be transported in xylem without difficulty. This data is in accordance with the results presented here.

The results obtained from the study of DCA metabolism in soybean (figures 3.5 and 3.6) suggested that within 30 min, DCA was taken up by the roots, conjugated to a malonyl-group and the conjugate released into the external medium. This requires: (1) a rapid uptake of [UL-^{14}C]-DCA by soybean roots. This is highly likely since log K_{ow} for DCA is 2.69. (2) A highly active DCA-*N*-MT, which was indeed observed (12,000 pkat g^{-1}, table 3.3) and (3) a transport system for the export of M-DCA. Since M-DCA is more hydrophilic than DCA, it is very unlikely that the conjugate could diffuse passively across the plasma membrane, back into the external medium. Furthermore, M-DCA is a weak acid (for its chemical structure,

see equation 1.7). The pK_a of M-DCA can be estimated as follows (Perrin *et al.*, 1981): pK_a of acetic acid is 4.7; this must be adjusted due to the presence of a $CONH_2$ group that increases the acid strength, hence 1.1 must be subtracted. Therefore, the pK_a for M-DCA approximates 3.6. Since the resting pH of plant cytosol is around 7.4 (Ryan and Walker, 1994), according to equation 3.1, over 99 % of the malonyl-conjugate would be dissociated to its anionic form, which in the absence of specific transporters would be retained within the cytoplasm due to ion trapping (figure 3.11 and Oparka, 1991; Sterling, 1994; Bromilow and Chamberlain, 1995).

$$pH = pK_a + \log\frac{[A^-]}{[HA]} \qquad \text{(Equation 3.1)}$$

Where HA is the undissociated acid and A^- is the dissociated, anionic form.

However, since the M-DCA concentration should be higher within the roots than in the medium (due to the large medium volume utilized in efflux experiments), one might argue that a very minor portion of unionised M-DCA could travel passively out across the plasma membrane, down its concentration gradient. The inhibitor experiment (figure 3.10) suggested that this is unlikely. Firstly, azide inhibited efflux by 10 %, suggesting a requirement for ATP. Azide does not completely deplete ATP in roots, since the alternative oxidase operates in anoxic conditions when the mitochondrial F1-ATPase is inactive. A treatment of salicylhydroxamic acid (SHAM) and cyanide has been shown to deplete root ATP completely (Blatt *et al.*, 1990) and might be expected to have a greater effect on efflux. Secondly, butyrate inhibited efflux by 30 %. Since butyrate decreases the cytoplasmic pH, the proportion of unionised M-DCA should be greater than at normal physiological pH; as a consequence, one would expect M-DCA efflux to increase, had passive diffusion been occurring. Since this was not observed, the possibility of a passive release of M-DCA through the plasma membrane can probably be ruled out. However, this result is not conclusive since the effect of butyrate on DCA-*N*-MT was not investigated. Nevertheless, it seems very likely that an efficient transport system is required for the efflux of M-DCA.

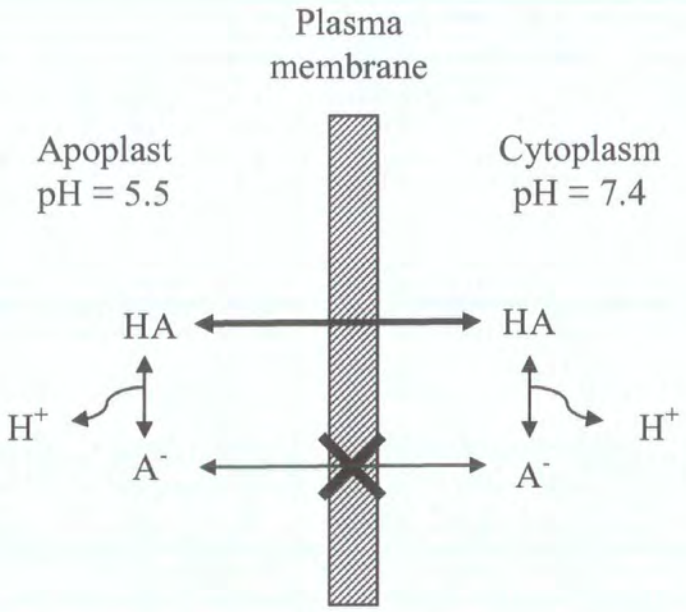


Figure 3.11: Accumulation of weak acids within cells, by ion trapping.

Weak acids cross the plasma membrane more readily in their unionised form AH, than in their anionic form A^-. In high pH compartments, weak acids are ionised to their anionic form A^-. Because A^- is more polar than its undissociated form AH, and because of its negative charge, it is unable to diffuse across the plasma membrane and therefore accumulates within the cytoplasm. Similarly, weak acids are exluded from the vacuole (pH < 5.5) by ion trapping in the cytoplasm.

In plants, several classes of transport systems can mediate the removal of toxic compounds from the cytoplasm, and detoxification can occur via at least two routes: export across the plasma membrane or sequestration in the vacuole. In recent years, vacuolar compartmentation has been most intensively studied and ABC-transporters were found to be involved in the vacuolar sequestration of negatively charged xenobiotic or natural metabolites whereas secondary transport systems are generally involved in the transport of both xenobiotic and endogenous glucosides. Plasma membrane efflux systems for endogenous metabolites and toxic compounds are much less well characterised but it is plausible that similar classes of transporters are involved. In fact, ABC-transporters of the MDR P-glycoprotein subfamily have been implicated in the efflux of both cytotoxic drugs (Thomas *et al.*, 2000) and auxins (Gaedeke *et al.*, 2001; Noh *et al.*, 2001) and PDR5 subfamily members have been shown to mediate the secretion of antifungal terpenoids (Jasinski *et al.*, 2001; van den Brule *et al.*, 2002). Since M-DCA is an organic anion, it might have been expected to be transported by the systems that accept xenobiotics and natural glucuronides. However, the results obtained here (section 3.2.3.2) provide preliminary evidence that a secondary transport system energised by the proton motive force, probably an organic anion-carrier, was involved. Time constraints prevented this observation from being followed up. In order to investigate the efflux mechanisms further, future experiments could include: first, a greater range of inhibitors and secondly, dose-response to inhibitors, as the concentrations used for this study were those normally applied to cell cultures. In fact, although the metabolism of DCA was studied using whole plants, it appears that the utilisation of single cells, protoplasts or vacuoles would have been valuable in order to investigate more precisely the transport mechanisms of DCA metabolites. Protoplasts, plant cells whose cell wall was removed, represent particularly good systems for the study of transport across the plasma membrane and would allow a greater reproducibility than that obtained with whole plant systems, mainly due to plant-to-plant variations. However, in order to eliminate energy-driven transport systems in other membranes and organelles (e.g. tonoplast, mitochondria, chloroplasts), studies using plasma membrane vesicles would be more appropriate. This latter approach does nevertheless present one fundamental limitation: many xenobiotic metabolites are sequestered in the vacuole and it is possible that M-DCA may be transported into this compartment. Therefore, to obtain information about each of the step of DCA

metabolism, the utilisation of both plasma membrane and tonoplast vesicles would be necessary.

The cross-feeding study presented in section 3.2.6.1 was designed to study the potential uptake of the DCA metabolites released by *Arabidopsis* and soybean roots. Figure 3.9 suggested that a small proportion of the DCA metabolites might be taken up by both plant species and possibly metabolised. However, both the uptake and re-export of conjugates synthesised *in planta* would require transporters and the input of energy, and would constitute a futile cycle for the plant. It seems more likely that M-DCA could diffuse from the growth medium into the apoplast, without re-entering the root symplast. Ideally, an uptake experiment using pure radiolabelled M-DCA and G-DCA would have been carried out but these were not readily available in sufficient quantities.

3.4. Summary

When the radiolabelled xenobiotic DCA was added to hydroponically-grown soybean plants, the toxic compound entered the roots passively and was rapidly and efficiently conjugated to a malonyl residue and released into the surrounding environment. The possible involvement of a pH-dependent organic anion-transporter was raised but a good deal of further work is necessary to support this. Some radiolabel was retained within the plant, mostly in the roots, both as soluble and non-extractable residues. The same general detoxification mechanism was observed in *Arabidopsis thaliana*, with a glucosyl-conjugate being the major exported compound although a small amount of M-DCA was also formed and exported. The detoxification routes for both soybean and *Arabidopsis* could be related to the relative activities of DCA-*N*-MT and DCA-*N*-GT within these plants.

Chapter 4

Purification of DCA-*N*-MT from soybean roots

4.1. Introduction

In chapter 3, the formation and export of an *N*-malonyl conjugate was confirmed as the major metabolic fate of DCA in soybean. In order to learn more about DCA metabolism, it was decided to isolate and purify DCA-*N*-MT. Although there have been attempts to purify DCA-*N*-MT (Matern *et al.*, 1984; Sandermann *et al.*, 1991), the molecular identity of this enzyme is currently unknown. Moreover, it is not clear to which protein family DCA-*N*-MT belongs and, since a facile genetic screen for the identification of DCA-*N*-MT was not available, a method based on its biochemical properties had to be adapted.

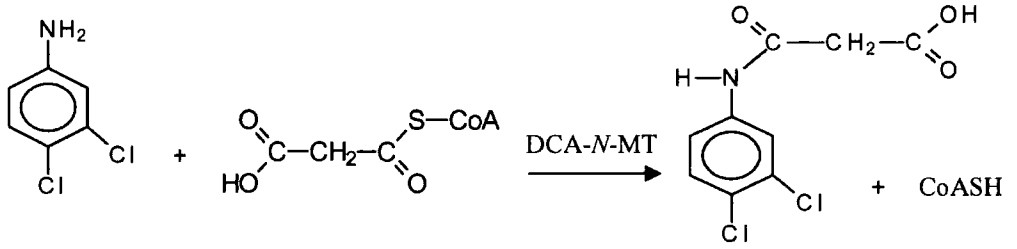
A purification scheme for DCA-*N*-MT has been reported for soybean suspension culture cells (Sandermann *et al.*, 1991). Therefore, it was decided to modify this protocol with the aim of purifying DCA-*N*-MT, in order to obtain peptide sequence data. As a first step, a robust and rapid assay was developed to monitor DCA-*N*-MT activity throughout the purification scheme.

4.2. Results

4.2.1. Development and optimisation of DCA-*N*-MT assay

Thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) combined with the utilisation of radiolabelled substrates

are techniques commonly used to assay enzyme activities during the metabolism of xenobiotics, but they are time consuming and can be expensive. In contrast, spectrophotometric assays are often simple, fast and cheap. Therefore, a coupled spectrophotometric assay was devised. Since DCA-*N*-MT catalyses the transfer of a malonyl group from malonyl-CoA onto DCA with the release of a CoA molecule (equation 1.7), the release of CoA could be determined using the reaction catalysed by α-ketoglutarate dehydrogenase (kDHase) which, in the presence of α-ketoglutarate and NAD leads to the production of succinyl-CoA and of $NADH_2$. The production of $NADH_2$ can be detected at 340 nm. However, a preliminary attempt to devise a coupled spectrophotometric assay based on these two reactions was unsuccessful. Therefore, it was decided to develop a radiometric assay, based on that published by Sandermann *et al.* (1991).



3,4-Dichloroaniline Malonyl-CoA M-DCA

(Equation 1.7)

4.2.1.1. Basis for DCA-N-MT assay

Sandermann and co-workers (1991) used the incorporation of radiolabelled DCA to assess DCA-*N*-MT activity in plant extracts. The reaction was performed in 100 mM potassium phosphate buffer, pH 6.5, containing 205 μM [UL-^{14}C]-DCA, 1 mM cold M-CoA and enzyme extract, in a final volume of 200 μl. The reaction was incubated at 40 °C for 40 min, stopped with the addition of 5 μl glacial acetic acid and the radioactive conjugate was extracted with 200 μl ethyl acetate. The compounds present in the solvent were then separated by TLC and radioactivity was detected and quantified by a linear TLC analyser (a schematic diagram is shown in figure 4.1).

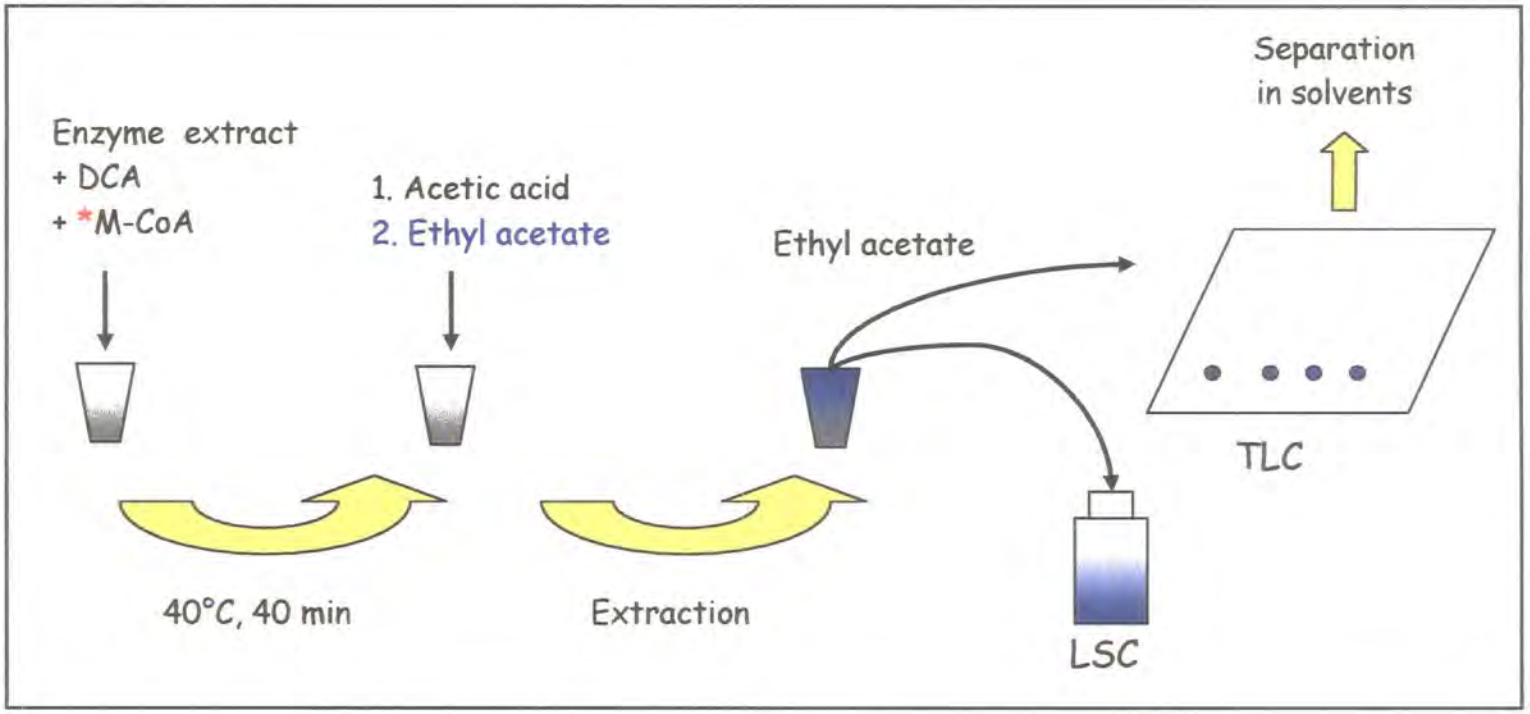


Figure 4.1: Principle of 3,4-Dichloroaniline- *N*-malonyltransferase assay.

3,4-Dichloroaniline (DCA) and [2-^{14}C]-Malonyl-Coenzyme A (*M-CoA) were mixed with enzyme extract and incubated at 40°C, for 40 min. The reaction was stopped with glacial acetic acid and malonyl-3,4-dichloroaniline was extracted in ethyl acetate, which was either spotted on a thin layer chromatography (TLC) plate or added to liquid scintillation counting (LSC) cocktail. Radioactivity was then quantified.

For this project, the assay was carried out with the following modifications:
(a) Both [UL-^{14}C]-DCA and [2-^{14}C]-M-CoA were tested as the source of radiolabel, i.e. [2-^{14}C]-M-CoA was used (final concentration of 19 μM, 200 nCi) with unlabelled DCA (205 μM) and [UL-^{14}C]-DCA (55 μM, 0.25 μCi) was used in conjunction with 150 μM unlabelled DCA and 100 μM unlabelled M-CoA (see reaction in equation 1.7).
(b) A proportion of the radiolabel was replaced with unlabelled compound in order to reduce the cost of the assay, since many assays were required to monitor protein purification.
(c) All the preliminary experiments were performed using TLC (section 4.1.2).
Initially, the identity and purity of reaction products were studied using a TLC linear analyser. However, this had the limitation that counting efficiency is only 10 % of the actual counts. By comparison, liquid scintillation counting (LSC, section 4.1.3) is a more sensitive technique and is also easier to perform, less time-consuming and more reproducible, since the ethyl acetate fraction is directly mixed with the liquid scintillation cocktail and the radioactivity quantified. The assay was therefore modified in several stages, to permit the quantification of M-DCA by LSC.

4.2.1.2. Qualitative analysis of the reaction products by TLC

In order to use LSC to measure DCA-*N*-MT, the reaction products must not be contaminated with radiolabelled substrate or by-product. Therefore, the ethyl acetate extracted reaction products were analysed by TLC and co-chromatographed with chemically synthesised M-DCA (section 2.1.3). Since this M-DCA standard was unlabelled, but absorbs UV light at 354 nm, the Rf values of radioactive peaks detected by the linear analyser were compared to those detected under UV light. In these experiments, the radiolabelled substrate employed was [2-^{14}C]-M-CoA. Two solvent systems were tested to optimise resolution and sample identification.

4.2.1.2.1. Solvent system 1

Solvent system 1 consisted of n-butanol/acetic acid/ water (8/1/1, v/v/v; Matern *et al.*, 1984). Co-chromatography of the samples with chemically synthesised M-DCA demonstrated that the conjugate had an Rf value of 0.92. A typical chromatogram obtained with solvent 1 is presented in figure 4.2.A. When a single radioactive peak was expected, two overlapping peaks were detected, at around Rf = 0.92. This observation may be due to the presence of acetic acid in the solvent mix, which caused the partial hydrolysis of the malonylated conjugate during the TLC migration (Winkler and Sandermann, 1992). Since M-DCA absorbs UV light, a photograph of the TLC plate at 354 nm was taken and clearly showed that solvent 1 gave a poor separation of the compounds throughout the plate (figure 4.2.B). Indeed, most molecules were taken all the way up to the front of the solvent. A second solvent system was therefore tested.

4.2.1.2.2. Solvent system 2

Solvent system 2 consisted of ethyl acetate/propan-2-ol/water (63/23/11, v/v/v; Sandermann *et al.*, 1991). The chromatogram obtained (figure 4.2.C) revealed a single peak (Rf = 0.34) corresponding to M-DCA (figure 4.2.D). Because the conjugate migrated in the first third of the plate, it was properly separated from other compounds (figure 4.2.D). Solvent system 2 was consequently applied to future experiments.

4.2.1.2.3. Choice of radiolabel

It was necessary to ensure that the radioactivity detected by LSC originated solely from the product of interest, i.e. M-DCA. Both [2-^{14}C]-M-CoA and [UL-^{14}C]-DCA were tested as radioactive substrates for DCA-*N*-MT assay. The chromatograms obtained are shown in figure 4.3. A single radioactive peak, corresponding to M-DCA, was detected when [2-^{14}C]-M-CoA was utilised (figure 4.3, panel A). When [UL-^{14}C]-DCA was used, two peaks were detected: one at Rf = 0.34, the other at Rf = 0.76, corresponding to M-DCA and DCA respectively (figure 4.3, panel B), demonstrating

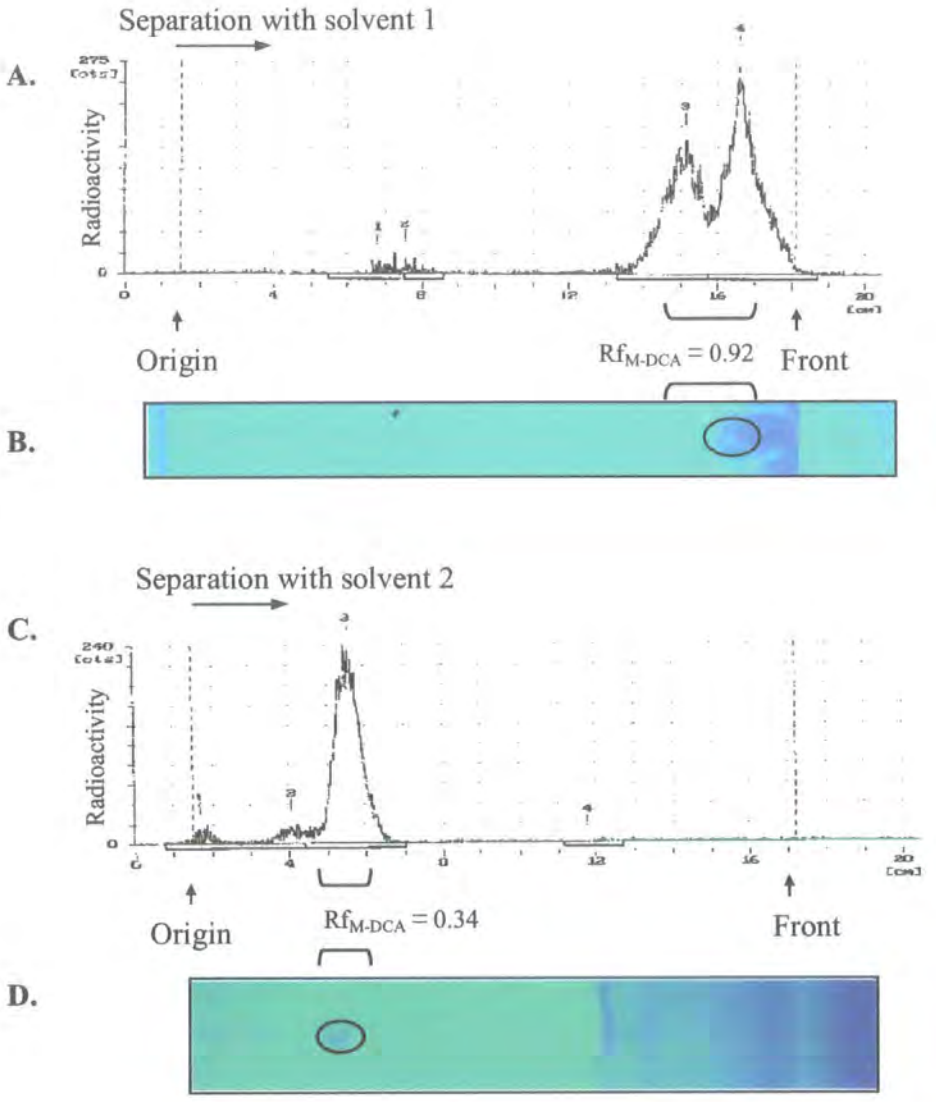


Figure 4.2: Assessment of two solvents for the separation of DCA-*N*-MT reaction products by TLC.

DCA-*N*-MT assay was performed with cold DCA and radiolabelled M-CoA. The reaction products were extracted with ethyl acetate and separated by TLC. **A** and **C:** chromatograms obtained from a TLC plate linear analyser. **B** and **D:** photographs of TLC plates taken under UV light (354 nm). **A and B:** Separation with solvent 1 (n-butanol:acetic acid:water; 8:1:1) led to the detection of 2 peaks, around Rf = 0.92. **C and D:** Separation with solvent 2 (ethyl acetate:propan-2-ol:water; 63:23:11) led to a single radioactive peak, at Rf = 0.34.

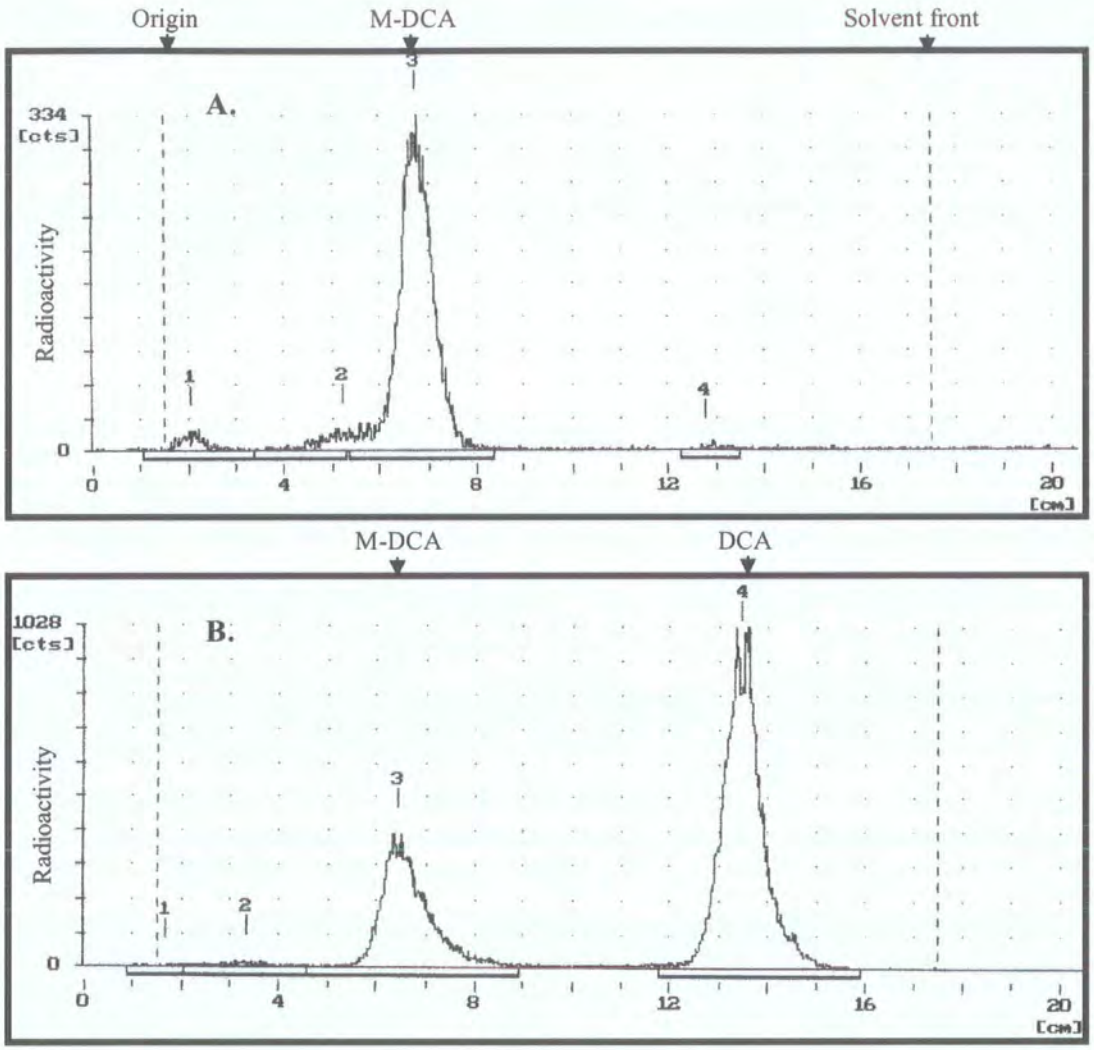


Figure 4.3: Selection of radiolabelled substrate for DCA-*N*-MT assay.

DCA-*N*-MT assay was performed with either [2-^{14}C]-malonyl-CoA or [UL-^{14}C]-3,4-dichloroaniline as the source of radiolabel, as described in section 4.2.1.1. Reaction products were extracted with ethyl acetate and analysed by TLC, in solvent 2 (ethyl acetate:propan-2-ol:water; 63:23:11). Radioactivity was detected using a linear analyser. Note the different scale on the y-axes. **Panel A:** [2-^{14}C]-malonyl-CoA was utilised as the radiolabelled substrate. A single, significant radioactive peak was detected at Rf = 0.34, corresponding to *N*-malonyl-3,4-dichloroaniline. **Panel B:** [UL-^{14}C]-3,4-dichloroaniline was utilised as the radiolabelled substrate. Two radioactive peaks were detected at Rf = 0.34 and Rf = 0.76, corresponding to *N*-malonyl-3,4-dichloroaniline and [UL-^{14}C]-3,4-dichloroaniline respectively.

the concomitant extraction of the radiolabelled DCA and M-DCA by ethyl acetate. Therefore, [2-^{14}C]-M-CoA was used in all further experiments.

4.2.1.3. Quantitative analysis of the reaction products by LSC

In order to ensure that the radiometric assay was quantitatively accurate, a quench curve was generated to compensate the loss of counts due to the presence of ethyl acetate (section 2.3.4.1) and the background reaction was tested. The number of counts obtained from reactions with boiled enzyme or without enzyme extract was negligible (data not shown), indicating that the radioactivity detected by LSC originated solely from the enzymatic synthesis of M-DCA.

4.2.1.4. Optimisation of DCA-N-MT assay

The standard reaction utilised to assay DCA-*N*-MT activity was performed at 40 °C, for 40 min, in the presence of DCA and [2-^{14}C]-M-CoA, in 100 mM potassium phosphate buffer, pH 6.5. However, preliminary experiments suggested that several assay parameters could be optimised. A comprehensive series of experiments was therefore conducted to determine the optimal pH, temperature, time, protein concentration and buffer conditions. Desalted ammonium sulphate precipitate extract from soybean roots was utilised for the optimisation of these assays.

4.2.1.4.1. Effect of temperature

Sandermann *et al.* (1991) performed their DCA-*N*-MT assay at 40 °C for 40 min, conditions under which the enzyme could be partially denatured. To determine whether these conditions were optimal, the standard reaction was carried out at 30 °C, 35 °C and 40 °C, for 40 min, with approximately 50 µg protein. DCA-*N*-MT activity was found to be highest at 35 °C (figure 4.4.A). DCA-*N*-MT assay was thereafter performed at 35 °C.

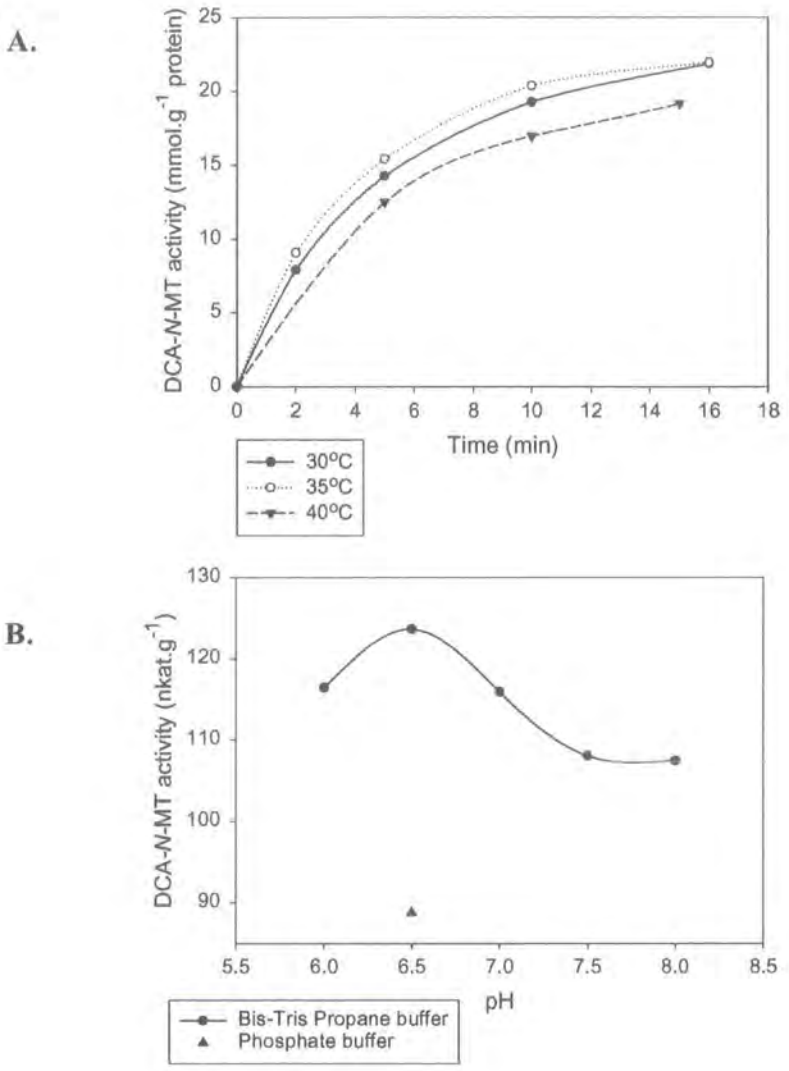


Figure 4.4: Effects of temperature, pH and buffer on DCA-N-MT activity in soybean roots.

After DCA-N-MT assay, radioactive compounds were extracted with ethyl acetate and quantified by LSC. **A.** Reaction carried out in the presence of 100 mM potassium phosphate buffer, pH = 6.5, with desalted root extract containing 50 µg protein. **B.** Reaction performed at 35 °C, for 2 min, with 0.1 % (w/v) BSA and either 100 mM potassium phosphate buffer (pH 6.5) or 100 mM Bis-Tris propane buffer (pH ranging from 6.0 to 8.0, with HCl). Desalted root extract containing 25 µg protein was utilised.

4.2.1.4.2. Effect of pH and buffer

The activity of DCA-*N*-MT was measured in 100 mM potassium phosphate buffer at pH 6.5 in the standard reaction. To test the effect of pH and buffer on DCA-*N*-MT activity, 100 mM Bis-Tris propane (1,3-bis[tris(hydroxymethyl) methylamino] propane), within a pH range of pH 6.0-8.0, was used. Bis-Tris propane was chosen because of its pK_a = 6.8 (at 25 °C), which is very close to the reported optimal pH for DCA-*N*-MT activity (Sandermann *et al.*, 1991). The activity obtained with the phosphate buffer and with the Bis-Tris buffer was compared. Approximately 25 µg protein was utilised and the reaction was performed at 35 °C, for 2 min, in the presence of 0.1 % (w/v) BSA. Results showed that the optimal pH to measure DCA-*N*-MT activity in the presence of Bis-Tris Propane was pH 6.5 (figure 4.4.B). Moreover, at this pH, the DCA-*N*-MT activity determined was about 50 % higher than that obtained when potassium phosphate buffer was used. It is well known that structurally different buffers interact specifically with reaction components (Blanchard, 1984). For example, phosphate buffers inhibit many kinases and dehydrogenases as well as carboxypeptidase, fumarase and urease. Bis-Tris propane possesses a different structure from phosphate which was found to be favourable to the DCA-*N*-MT assay studied.

4.2.1.4.3. Effect of bovine serum albumin (BSA)

It is generally believed that the presence of additional proteins in an enzyme extract will have a protective effect on enzymatic activity. The rationale behind this fact is that the additional proteins will buffer the effect of adverse conditions (e.g. protein inhibitors, oxidative agents) thus reducing the damage to the enzyme of interest. For this experiment, the effect of 0.1 % (w/v) BSA (final concentration) on DCA-*N*-MT activity was studied, using desalted root extract with and without BSA. The reaction was carried out in 0.1 M Bis-Tris Propane buffer, pH 6.5, at 35 °C, for 5 min. The presence of 0.1 % BSA increased the enzymatic activity measured by up to 3-fold (figure 4.5).

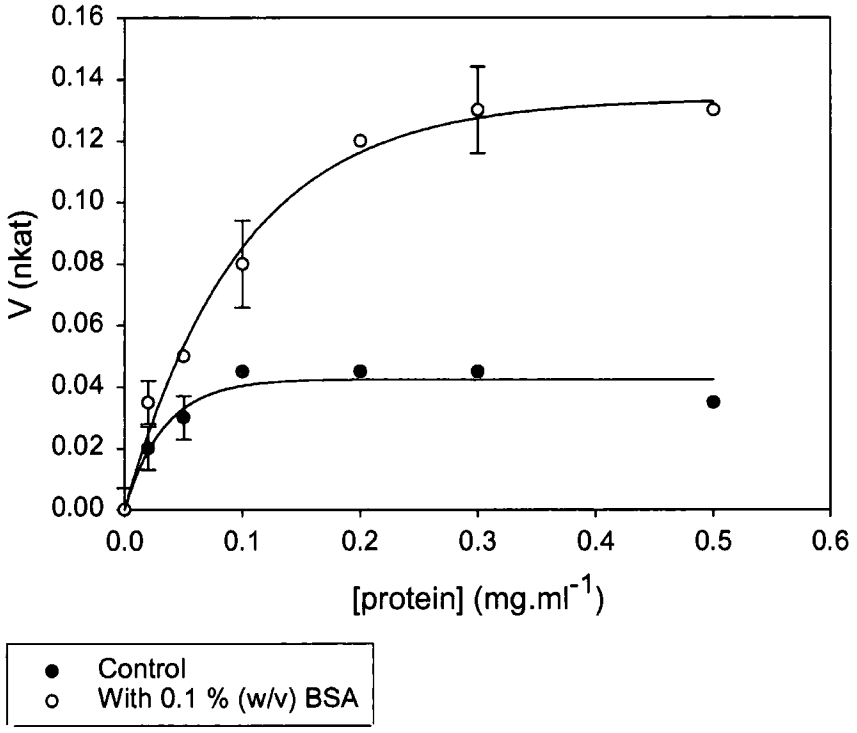


Figure 4.5: Effect of 0.1 % (w/v) bovine serum albumin (BSA) on DCA-*N*-MT activity in soybean roots.

Assay was conducted at 35 °C for 5 min, with 100 mM potassium phosphate buffer, pH 6.5. The radioactive compounds were extracted with ethyl acetate and quantified by LSC.

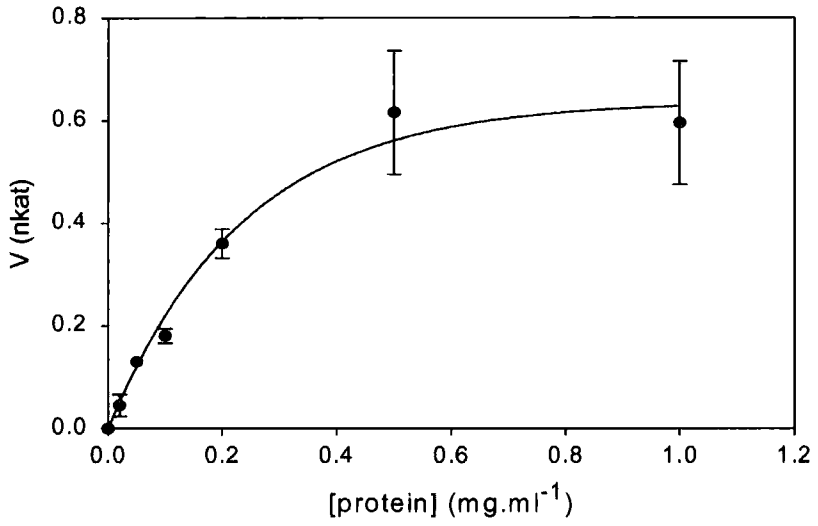
4.2.1.4.4. Linearity of the assay

The protein and time dependence of the DCA-*N*-MT assay were studied, to determine the conditions necessary for the assay to be linear. Based on the results presented above, the basic assay was modified. The reaction was thereafter carried out at 35 °C, in 100 mM Bis-Tris propane, pH 6.5, with 0.1 % (w/v) BSA. Under these conditions, the assay was linear for 5 min, with ≤ 0.2 mg protein ml^{-1}, when soybean root extract was used (figure 4.6). In the case of soybean suspension-cultured cells, the assay was linear in the same conditions, but only up to 0.1 mg protein ml^{-1} (figure 4.7). Since DCA-*N*-MT activity is much higher in cell cultures than in roots (section 5.2), this observation was expected.

4.2.2. Purification procedure

Since a partial purification procedure had been published, it was used as a starting point for the present study. Using suspension-cultured soybean cells as starting material, Sandermann and co-workers (1991) used a six-step scheme and obtained a 74-fold purification of DCA-*N*-MT, with a 10 % yield. First, the cells were frozen in liquid nitrogen and ground to a powder using a mortar and pestle and 1.5 (v/w) buffer 1 (200 mM Tris-HCl, pH = 7.5, 2 mM $MgCl_2$, 2 mM DTT, 1 mM PMSF) was added. The extract was then filtered through Miracloth and the filtrate was cleared by centrifugation. Step 2: proteins in the supernatant thus obtained were precipitated with finely powdered ammonium sulphate. Proteins precipitated between 35-80 % (w/v) were desalted on a Sephadex G-25 column, in buffer 2 (20 mM Tris-HCl, pH = 7.5, 2 mM $MgCl_2$, 2 mM DTT, 1 mM PMSF). Step 3: the desalted solution was applied to a DEAE-Sepharose column and elution was with 2 linear gradients, one between 0 and 400 mM NaCl in buffer 2 and the other with 400 mM to 2 M NaCl in buffer 2. Active fractions eluted between 200 and 400 mM NaCl were concentrated with 80 % (w/v) ammonium sulphate and re-dissolved in a small volume of buffer 1. Step 4: protein extract was applied to a Sephadex G-100 column for a gel permeation chromatography. The active fractions were concentrated with 80 % (w/v) ammonium sulphate and re-

A.



B.

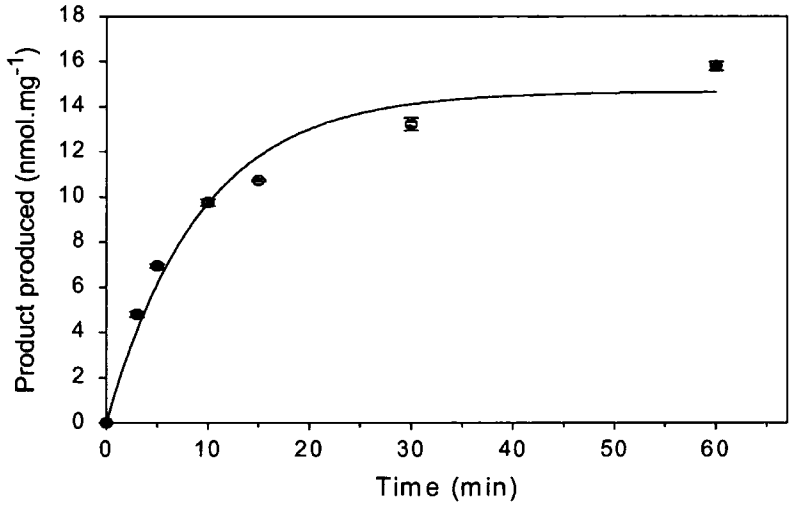
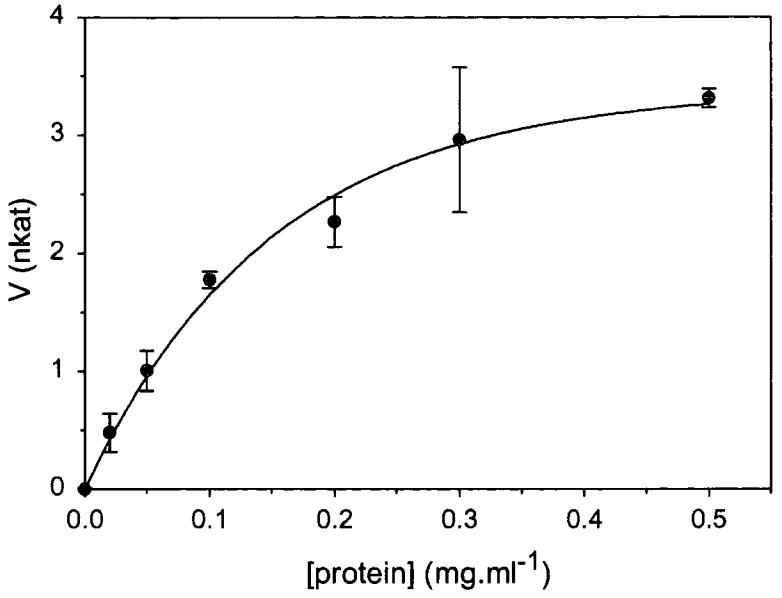


Figure 4.6: Linearity of DCA-*N*-MT assay, with respect to protein concentration and time.

DCA-*N*-MT assay was performed at 35 °C, in 100 mM Bis-Tris propane buffer, pH 6.5, with desalted soybean root extracts. The reaction was carried out for 5 min (**A**) while the protein concentration was varied and (**B**) with 0.2 mg ml^{-1} protein, while the incubation time was varied. The radioactive compounds were extracted with ethyl acetate and quantified by LSC.

A.



B.

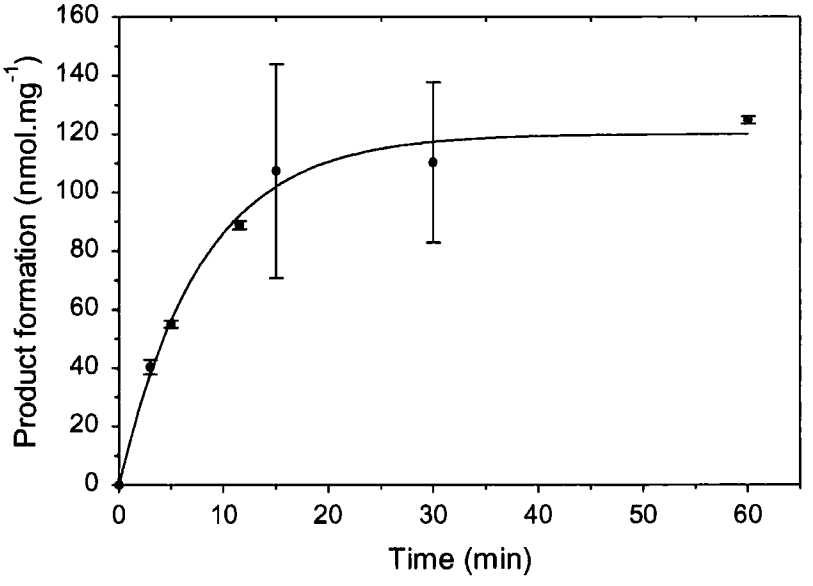


Figure 4.7: Linearity of DCA-*N*-MT assay with cultured cell extract, with respect to protein concentration and time.

DCA-*N*-MT assay was performed at 35 °C, in 100 mM Bis-Tris propane buffer, pH 6.5, with desalted soybean suspension cultured cell extracts. The reaction was carried out for 5 min (**A**) while the protein concentration was varied and (**B**) with 0.1 mg ml^{-1} protein, while the incubation time was varied. The radioactive compounds were extracted with ethyl acetate and quantified by LSC.

dissolved in a small volume of buffer 1. Step 5: protein extract was applied to a phenyl-Sepharose CL-4B column and elution was with a linear gradient of 0-60 % ethylene glycol in buffer 1. Active fractions came through the column directly and proteins were concentrated by precipitation with 75 % (w/v) ammonium sulphate, re-dissolved in buffer 3 (100 mM potassium phosphate, pH = 6.5) and dialysed overnight against buffer 3. Step 6: the protein solution was applied to a gel permeation Ultrogel AcA 54 column in buffer 3 and DCA-*N*-MT eluted with an apparent molecular weight of 48,000.

Based loosely on this protocol, two 6-step purification schemes were developed (figure 2.3) which led to an overall 400-fold purification and 1.7 % yield. In order to reduce the possibility of protein degradation and inactivation, Sephadex G-25 columns were used to desalt protein extracts, in place of the overnight dialysis favoured by Sandermann and co-workers.

4.2.2.1. Choice of starting material

A range of plant material was tested to determine the best source of soybean DCA-*N*-MT for purification. However, this was performed before the DCA-*N*-MT assay was completely optimised. Thus the reaction mixture consisted of crude root and cell extracts (5 µg and 3 µg protein, respectively), with 205 µM DCA, 19 µM [2-^{14}C]-malonyl-coA and 0.1 % BSA in 100 mM potassium phosphate, pH 6.5, to a final volume of 200 µl. The reaction was performed at 35 °C for 2 min.

DCA-*N*-MT activity in crude cell extracts was measured in 7 to 22 d-old cells. During the exponential growth phase (figure 2.1), from 7 d to 16 d, the average DCA-*N*-MT activity was 235.7 ± 11.7 nkat g^{-1}, which was 2 to 11-fold higher than that in roots of the same age. This value decreased to 163 ± 19 nkat g^{-1} at 22 d at the end of the stationary phase, probably due to the increase in cell death.

The roots and shoots were analysed separately. DCA-*N*-MT activity in soybean shoots was found, in previous experiments, to be approximately 40-fold lower than that in the roots (table 3.3). In the roots, DCA-*N*-MT activity increased with the age of the plant, from 19.9 ± 3.7 nkat g^{-1} at 7 d to 119.1 ± 15.1 nkat g^{-1} at 17 d (table 4.1). Therefore, it seemed logical to use 17 d-old roots for DCA-*N*-MT purification.

Root age (d)	Specific Activity (nkat g^{-1} protein)
7	19.9 ± 3.7
11	34.7 ± 3.9
14	61.7 ± 16.8
17	119.1 ± 15.1

Table 4.1: Developmental profile of DCA-*N*-MT activity in soybean roots.
Soybean seedlings were grown in vermiculite at 20 °C (16 h photoperiod) for up to 17 d. At different ages, proteins were extracted from the roots, as described in section 2.4.1. DCA-*N*-MT activity was determined as described in section 2.3.8.1, except that the assay was performed at 35 °C, for 2 min. Values represent means ± standard deviation of three replicates.

However, it proved very difficult to rinse vermiculite from older roots, as opposed to younger ones. A compromise between the age of the roots utilised, and thus their level of DCA-*N*-MT activity, and the contamination of the starting material with vermiculite had to be reached. Eventually, it was decided to attempt DCA-*N*-MT purification using 14 d-old soybean roots. Although suspension cultures were the richest source of DCA-*N*-MT activity, it proved difficult to grow sufficiently large quantities of cells.

In order to assess the future possibility of using the wide range of *Arabidopsis* genomic resources to identify and isolate the gene(s) encoding DCA-*N*-MT, crude protein extracts from *Arabidopsis* plants and tissue cultures were tested for DCA-*N*-MT activity. No DCA-*N*-MT activity was detected in extracts from mature, whole plants and roots. Very low activity was detected in suspension cultured cells (98 pkat g^{-1}) and in root cultures (52 pkat g^{-1}). The hope that *Arabidopsis* could represent an alternative and easier way of cloning our enzyme of interest, DCA-*N*-MT, was therefore abandoned.

4.2.2.2. Purification procedure 1

4.2.2.2.1. Crude extracts

Crude protein extracts were obtained from soybean tissue following the protocol published by Sandermann *et al.,* 1991 (described in section 2.4.1). This crude extract being very dilute, the proteins were concentrated by ammonium sulphate precipitation. A flow chart of the purification steps is given in figure 2.3. To determine the optimal range of ammonium sulphate to use, i.e. that which yields the highest DCA-*N*-MT activity with the smallest amount of contaminating protein, proteins were precipitated with 10-80 % saturation ammonium sulphate (10 % stepwise increase). After each precipitation, the protein concentration and DCA-*N*-MT activity were assessed and expressed as the percentage of total protein and DCA-*N*-MT activity recovered. As shown in figure 4.8, approximately 96 % DCA-*N*-MT activity and 84 % protein were recovered between 40-70 % ammonium sulphate saturation. Below 40 %, approximate-

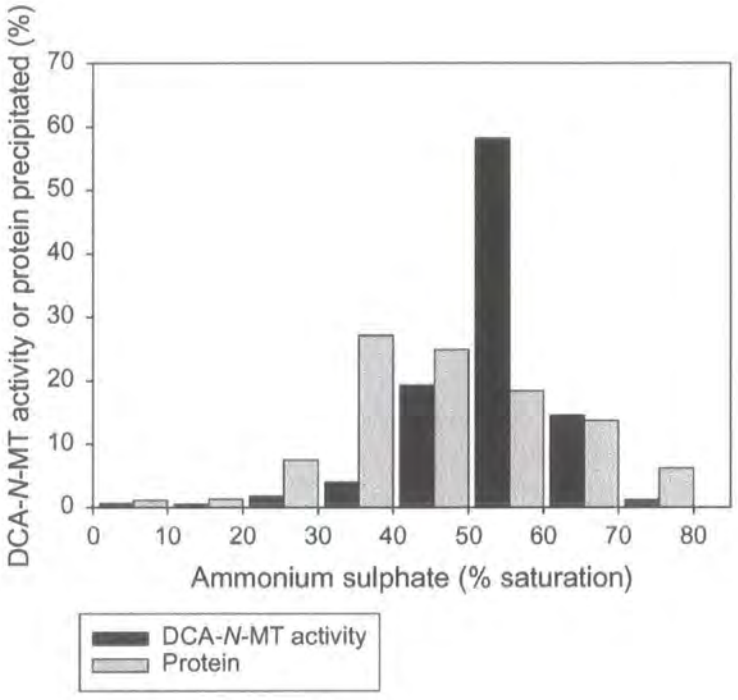


Figure 4.8: Recovery of DCA-*N*-MT during ammonium sulphate precipitation.
Proteins in crude root extracts were precipitated with increasing amounts of finely-powdered ammonium sulphate (10 % saturation, stepwise gradient). At each step, the precipitated protein was analysed for protein content and DCA-*N*-MT activity as described in sections 2.4.4 and 2.3.8.1, respectively. The protein content and DCA-*N*-MT activity at each step were expressed as the percentage of the total protein and DCA-*N*-MT activity recovered during the experiment.

ly 3 % DCA-*N*-MT activity was recovered, with 10 % protein. Above 70 % ammonium sulphate, these figures changed to 1 % and 6 %, respectively. Following these observations, ammonium sulphate precipitation was carried out between 40 % and 70 %. The resulting pellet was desalted using a Sephadex G-25 column (PD10 column, Amersham Biosciences). At this stage, a 1.2-fold purification of the enzyme of interest was observed. This value was consistent with data published by Sandermann and co-workers (1991), who used 35-80 % (w/v) ammonium sulphate.

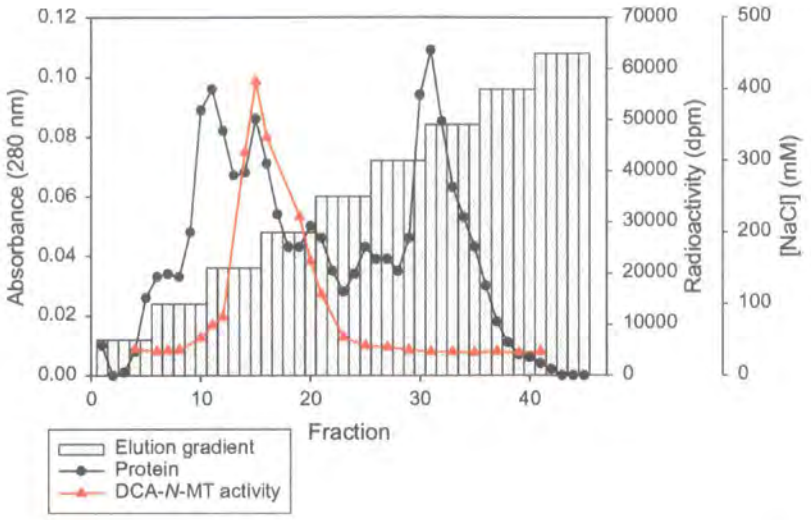
4.2.2.2.2. Centricon filters

Centricon filters were used to concentrate protein extracts after desalting the ammonium sulphate precipitate and in order to achieve crude size exclusion. In practice, desalted extract was filtered through the Centricon filter with the higher cut-off point (100 or 50 kDa) and the filtrate was filtered a second time on a filter with a lower cut-off point (50 or 30 kDa). Combinations of filters with various cut-off points (30, 50 and 100 kDa) were tested. Unfortunately, the results obtained were not reproducible therefore, proteins retained between 30 and 100 kDa were used to ensure that most of the DCA-*N*-MT was recovered. Although this step allowed a three-fold enzyme purification, it retained only 6 % of the initial DCA-*N*-MT activity (see below).

4.2.2.2.3. Ion exchange chromatography

Following the protocol of Sandermann and co-workers (1991), an ion exchange step employing a diethylaminoethyl (DEAE) anion-exchanger was tested. Protein extracts were loaded onto this DEAE-Sepharose column in a low salt buffer (pH 7.5) and were eluted with a NaCl step gradient in the same buffer, containing from 0 to 450 mM NaCl (figure 4.9.A). The peak of DCA-*N*-MT activity was eluted between 150 and 200 mM NaCl. Consequently, in subsequent experiments, the elution range was narrowed to 100-300 mM NaCl, with the gradient incrementing by 50 mM NaCl with each step. Most DCA-*N*-MT activity was eluted with 200 mM NaCl (figure 4.9.B). This

A.



B.

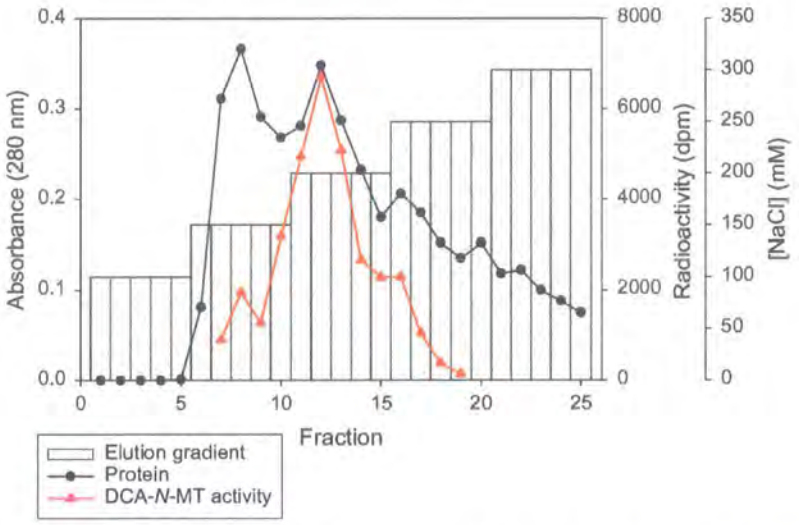


Figure 4.9: Elution profile of DCA-*N*-MT from a DEAE-Sepharose column.

Soybean root protein extract concentrated on Centricon filters was applied to a DEAE-Sepharose column, in 20 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, and eluted with a stepwise NaCl gradient in the same buffer, at 4 ml min^{-1}. One ml fractions were collected. The protein content of each fraction was assessed by spectrophotometry at 280 nm and DCA-*N*-MT assay was as described in section 2.3.8.1. **A.** In initial experiments, an elution gradient of 0-450 mM NaCl in buffer was applied. **B.** In later experiments, a more narrow elution gradient of 100-300 mM NaCl in buffer was applied.

ion exchange chromatograph allowed a six-fold purification of DCA-*N*-MT and an overall 20-fold purification was achieved, which was surprisingly high compared to the 2.6-fold purification reported by Sandermann and co-workers (1991). The difference between these values could possibly be explained by the broad activity peak (between 200 and 400 mM NaCl) that they pooled, compared to the relatively sharp elution peak obtained here, at 200 mM NaCl (figure 4.9.B).

4.2.2.2.4. Hydrophobic interaction chromatography

After DEAE-Sepharose, Sandermann and co-workers (1991) used a gel filtration step in the form of a Sephadex G-100 column. Since a crude size exclusion step had already been introduced early on in this purification scheme, with the Centricon filters, a gel filtration step was omitted and active fractions obtained from the DEAE-Sepharose column were directly loaded onto a Phenyl-Sepharose column. Sandermann and co-workers (1991) applied their protein extract in a buffer containing no salts, which is probably why the enzyme of interest did not bind to the phenyl-Sepharose column and was rinsed off directly. Consequently, to promote the binding of DCA-*N*-MT by hydrophobic interaction, a buffer containing 15 % (w/v) ammonium sulphate was used as starting buffer. Proteins were subsequently eluted with buffer containing decreasing amounts of salts. The peak of DCA-*N*-MT activity was eluted with buffer without salt (figure 4.10). This hydrophobic interaction chromatography allowed a six-fold purification and an overall 100-fold purification of DCA-*N*-MT was achieved (see below), which was more than two-fold better than the published data (Sandermann *et al.*, 1991).

4.2.2.2.5. Dye-ligand chromatography

A dye-ligand chromatography step was added after the hydrophobic interaction chromatography in order to increase the purification of DCA-*N*-MT. Assuming that a dye specific for our enzyme of interest could be found, dye-ligand chromatographies could theoretically achieve as good a purification as a conventional affinity column

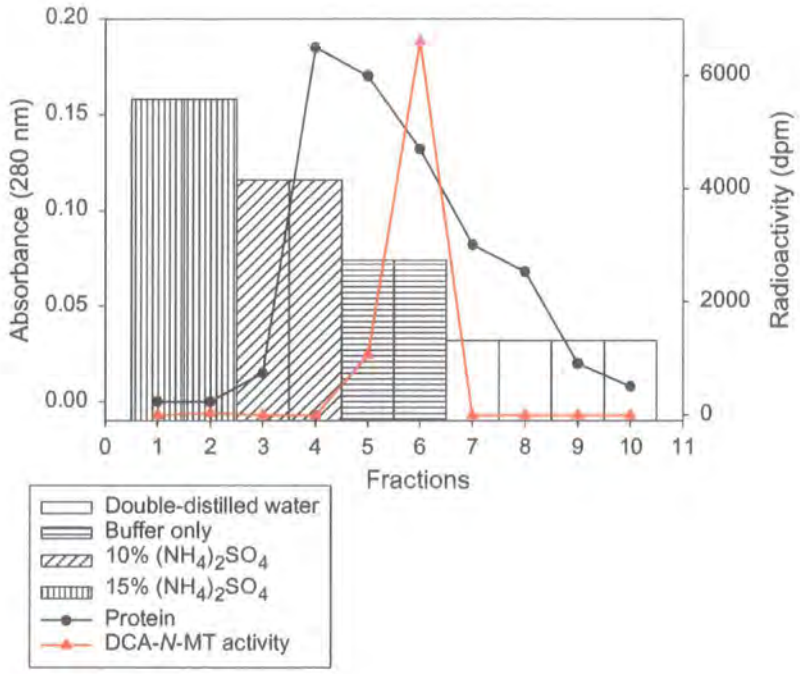


Figure 4.10: Elution profile of DCA-*N*-MT from a phenyl-Sepharose column.

Active fractions eluted from DEAE-Sepharose chromatography were applied in 15 % (w/v) ammonium sulphate, in start buffer (20 mM Tris-HCl, pH = 7.5, 2 mM $MgCl_2$). Proteins were eluted with buffer containing decreasing amounts of ammonium sulphate and then with double-distilled water. Flow rate: 1 ml min^{-1}. One ml fractions were collected. The protein content of each fraction was assessed by spectrophotometry at 280 nm and DCA-*N*-MT assay was as described in section 2.3.8.1. dpm: disintegration per minute.

(Stellwagen, 1990). Initially, a series of different dye-columns were screened using desalted ammonium sulphate precipitate. Protein extracts were loaded onto each column, unbound protein was rinsed off with 5 ml buffer, and protein was eluted with 7.5 ml buffer containing 1 M NaCl. 2.5 ml fractions were collected. According to the elution profiles of DCA-*N*-MT, the dye-columns were separated in two groups (table 4.2): (1) columns that did not bind DCA-*N*-MT (known as negative columns) and (2) columns that bound DCA-*N*-MT (known as positive columns). With the exception of Blue 4 dye matrix, from which only 35 % DCA-*N*-MT was recovered, the total DCA-*N*-MT activity obtained from all the dye-ligand columns was 1.5 to 3.7-fold higher than that loaded, suggesting the possible removal of molecules inhibiting DCA-*N*-MT activity. Although most protein was recovered from the negative columns, less than 60 % protein was recovered from the positive columns, suggesting a strong binding between the protein applied and the dye-matrix, and thus a potentially difficult regeneration of the columns. The negative column that retained most unwanted proteins contained Blue 72 dye whereas the best two positive dye matrices were Cibacron blue F3G-A and Green 19.

The best negative dye, Blue 72, and one of the best positive dyes, Cibacron Blue F3G-A, were studied further. Cibacron Blue F3G-A was chosen over Green 19 because it had been used for the successful purification of 1-aminocyclopropane-1-carboxylate-*N*-MT from etiolated mung bean hypocotyls (Guo *et al.*, 1992; Guo *et al.*, 1993) and from tomato fruit (Martin and Saftner, 1995), and for the partial purification of D-tryptophan-*N*-MT from tomato leaves (Wu *et al.*, 1995). Three ml desalted ammonium sulphate extract of soybean roots was loaded on both columns, unbound protein was rinsed off with 7 ml buffer and elution was with 8 ml buffer containing 1 M NaCl. One ml fractions were collected and assessed for their protein content and DCA-*N*-MT activity. 73.9 % DCA-*N*-MT activity and 46.6 % of protein was eluted with the Cibacron Blue F3G-A column (figure 4.11). With Blue 72, the corresponding values were of 89 % and 73 %, respectively. Therefore, Cibacron Blue F3G-A column was chosen as the dye-ligand chromatography step. Note that first, more than 60 % DCA-*N*-MT activity and 28 % protein were eluted simultaneously from the Cibacron Blue F3G-A column (figure 4.11). This dye-ligand step could therefore allow at least a two-fold

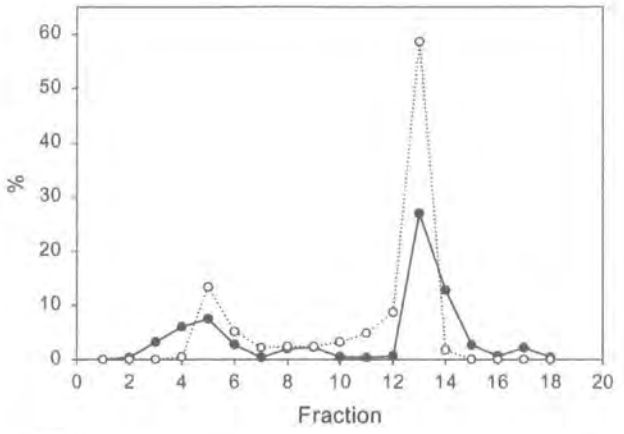
Positive dye-columns		Blue 4	Yellow 3	Blue F3G-A	Green 19
% protein	Unbound	17.6	17.8	20.1	19.7
	Eluted	27.4	40.7	34.0	27.2
	Total	45.0	58.5	54.1	47.0
% activity	Unbound	9.2	15.3	20.1	9.1
	Eluted	25.4	135.4	166.1	154.4
	Total	34.6	150.7	186.2	163.5

Negative dye-columns		Red 120	Brown 10	Yellow 86	Blue 72	Green 5
% protein	Unbound	54.6	75.9	76.0	51.1	52.1
	Eluted	24.2	18.1	17.1	47.1	33.6
	Total	78.7	94.0	93.0	98.2	85.7
% activity	Unbound	132.3	292.8	268.9	323.8	334.5
	Eluted	104.3	20.9	17.2	38.2	38.1
	Total	236.6	313.7	286.1	362.1	372.6

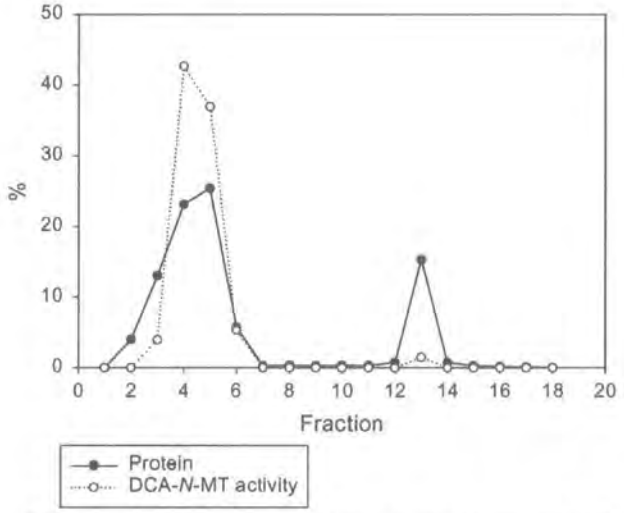
Table 4.2: Comparison of dye-ligand chromatography columns for DCA-*N*-MT purification.

Desalted root extract (2 mg protein, 0.13 nkat) was loaded onto each column. The columns were rinsed with 2 x 2.5 ml buffer (20 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 5 mM DTT) to eliminate unbound proteins ("rinsed off"). Proteins bound to the column were then eluted with 3 x 2.5 ml of 1 M NaCl in the same buffer. 2.5 ml fractions were collected and assessed for protein content and DCA-*N*-MT activity. Positive columns are defined as those which bind DCA-*N*-MT and negative ones as those which do not. Figures are given as percentages of the initial protein dose and DCA-*N*-MT activity loaded.

A.



B.



C.

Dye-columns		Blue F3G-A	Blue 72
% protein	Unbound	24.9	72.8
	Eluted	46.6	17.8
	Total	71.5	90.6
% activity	Unbound	29.1	88.9
	Eluted	73.9	1.5
	Total	103.0	90.4

Figure 4.11: Elution of DCA-*N*-MT from dye-ligand columns.

Desalted root extract (7.5 mg protein, 0.38 nkat, in 3 ml) was loaded onto a Cibacron Blue F3G-A (**A**) or a Blue 72 column (**B**). The columns were rinsed with 7 ml buffer (20 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 5 mM DTT). Proteins were eluted with 1 M NaCl in the same buffer. One ml fractions were collected and assessed for their protein content and DCA-*N*-MT activity. Figures are given as percentages of the protein dose and DCA-*N*-MT activity loaded.

purification. Secondly, the results obtained here differ from those presented in table 4.2. Previously DCA-*N*-MT activity was up to three-fold higher after its passage through the dye-columns. However, when this was repeated, the total DCA-*N*-MT activity recovered from the Cibacron Blue F3G-A and the Blue 72 columns was 103 % and 90.4 %, respectively (figure 4.11). Later experiments showed that it was difficult to regenerate these columns, suggesting that had there been a removal of inhibitory molecules, this was possibly lost upon the re-utilisation of the columns. Thirdly, as mentioned above, the positive dye columns studied bound proteins very strongly, which rendered the regeneration of the column very difficult. In fact, when first used, only 54 % of the protein loaded was recovered from the Cibacron Blue F3G-A. This figure increased to 70 % in later experiments (figure 4.11) but very rapidly, the column had to be replaced with a new one because it had lost its specificity for DCA-*N*-MT. This was likely due to the incomplete regeneration of the matrix, even though the manufacturer's protocol was scrupulously followed.

When the Cibacron Blue F3G-A chromatography was added to the purification scheme, Coenzyme A was tested as specific counter-ligand to effect elution of DCA-*N*-MT but without success (data not shown). A stepwise gradient of NaCl was therefore applied for the elution of DCA-*N*-MT. The enzyme was eluted between 0.2-0.4 M NaCl (figure 4.12). Unfortunately, the protein concentration of the active fraction was too low to be detected with the Bradford assay; as a consequence, the specific activity of the enzyme could not be determined. The degree of purification at this stage was thus estimated on the basis of the preliminary experiments described above. This showed that DCA-*N*-MT could be purified at least two-fold when a loaded on a Cibacron Blue column, after a phenyl-Sepharose column, but according to DCA-*N*-MT elution profile of the larger scale Cibacron Blue F3G-A (figure 4.12), the purification of the enzyme during this chromatography step should be higher.

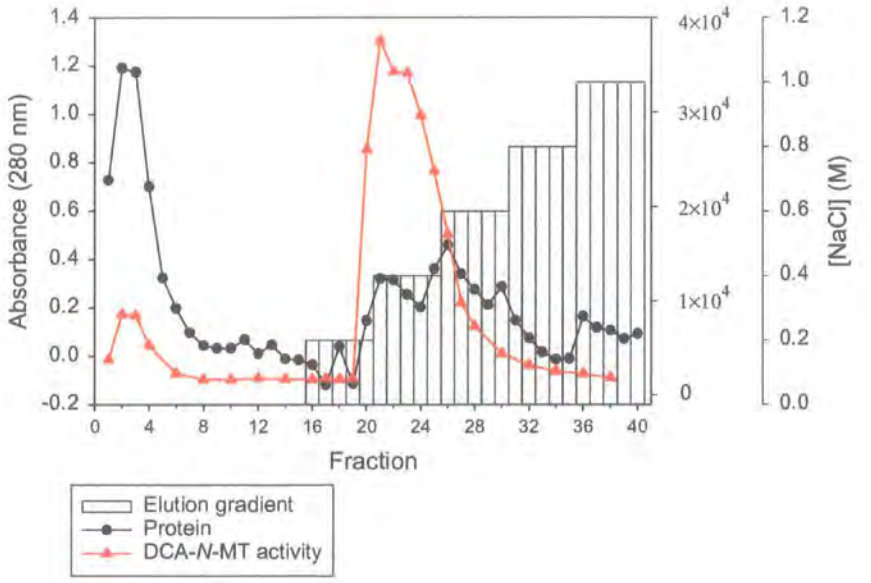


Figure 4.12: Elution profile of DCA-*N*-MT from a Cibacron Blue F3G-A column. Active fractions obtained from the phenyl-Sepharose column were applied to a Cibacron Blue F3G-A column in 20 mM Tris-HCl, pH = 7.5, 2 mM $MgCl_2$, and eluted with a stepwise gradient of NaCl in the same buffer, at 4 ml min^{-1}. One ml fractions were collected. The protein content of each fraction was assessed by spectrophotometry at 280 nm and DCA-*N*-MT assay was as described in section 2.3.8.3. Dpm: disintegrations per minute.

4.2.2.2.6. Assessment of purification procedure 1

A balance sheet for the purification of 17 d-old soybean roots (approximately 100 g) is given in table 4.3. The utilisation of Centricon filters caused the loss of most DCA-*N*-MT activity (more than 90 % of the starting activity). It was therefore crucial to eliminate their utilisation. Despite the low loading, there was an overall 20-fold purification of the enzyme after the DEAE-Sepharose step. The subsequent hydrophobic interaction step led to a further five-fold purification, giving an overall 100-fold purification. Unfortunately, there was not enough protein left after the Cibacron blue F3G-A chromatography to determine the specific activity of the fraction. Although this purification was repeated several times with more starting material, it was not possible to obtain enough protein for analysis, probably due to the earlier loss caused by the Centricon filters. A final DCA-*N*-MT purification of at least 200-fold was nevertheless estimated, based on preliminary experiments with the Cibacron blue F3G-A column.

To assess this purification scheme further, the active fractions from the crude extract and from the phenyl-Sepharose chromatography were analysed by SDS-PAGE (figure 4.13). After the phenyl-Sepharose column, 5 bands with apparent molecular weights of 110, 57, 53, 45, 27 and 20 kDa, appeared to be enriched by the purification. Sandermann *et al.* (1991) extracted a DCA-*N*-MT from suspension-cultured soybean cells and reported the enzyme to be monomeric, with a molecular weight of 48 ± 3 kDa, as determined by gel permeation chromatography and under denaturing conditions using SDS-PAGE. Another DCA-*N*-MT from peanut leaves and hypocotyls was found to be 45 kDa, as determined by gel permeation chromatography (Matern *et al.*, 1984). If our DCA-*N*-MT was similar to these enzymes, the 53 kDa and the 45 kDa proteins would be good candidates. Since purification procedure 1 led to a very low recovery of DCA-*N*-MT and did not allow the purification of DCA-*N*-MT to homogeneity, an alternative protocol that would give better yield and purification was tested.

	Total protein (mg)	*SA (nkat.g^{-1})*	*Purification (fold)*	*Yield (%)*
Crude	50.0	136.6	1.0	100.0
$(NH_4)_2SO_4$	17.8	160.1	1.2	41.7
Desalted	28.0	150.2	1.1	61.6
Centricon	1.1	396.6	2.9	6.4
DEAE-Sepharose	0.1	2709.8	19.8	4.0
Phenyl-Sepharose	0.006	13820.0	101.2	1.2

Table 4.3: Balance sheet for the purification of DCA-*N*-MT following procedure 1. Crude extract from 17 d-old soybean roots (100 g) was treated with 40-70 % saturation ammonium sulphate. The precipitate was desalted using a Sephadex G-25 (PD10) column and filtered through Centricon filters. Proteins retained between 30 and 100 kDa cut-off filters were applied to a DEAE-Sepharose column. Active fractions were then loaded onto a phenyl-Sepharose column. SA: specific activity.

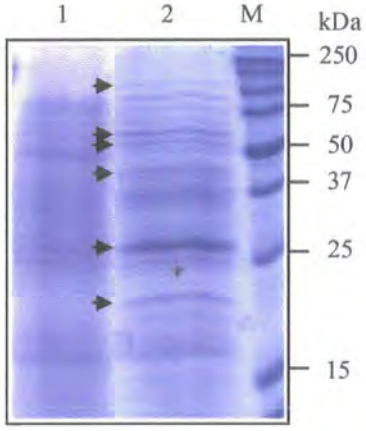


Figure 4.13: SDS-PAGE of DCA-*N*-MT during purification procedure 1.

Samples (4 μg) from active fractions were separated in a 13.5 % SDS-polyacrylamide slab gel and stained with Coomassie blue. 1, crude extract; 2, phenyl-Sepharose; M, molecular weight marker. The arrows indicate the bands that were enriched during the purification. Their apparent size was: 110, 57, 53, 45, 27 and 20 kDa.

4.2.2.3. Purification procedure 2

Based on the previous purification scheme, a second procedure was tested with several modifications. Firstly, the Centricon filter step was eliminated as it caused high losses of DCA-*N*-MT. Secondly, linear elution gradients were applied to all the columns. Step gradients cause proteins to be eluted with a rapid increase of salts, which usually leads to a sharp elution of the enzyme of interest but may result in the concomitant elution of unwanted proteins. In contrast, linear gradients allow a slower elution of proteins. Although this potentially leads to broader enzyme activity peaks, the levels of contaminating proteins can be reduced. These modifications were applied to purification procedure 1 as described below and additional steps were tested.

4.2.2.3.1. Protamine sulphate precipitation

As mentioned in section 4.2.2.6, the utilisation of Centricon filters had to be eliminated. However when this concentration step was omitted, the protein extract could not be filtered through a 0.42 μm pore size filter, which is a routine step to prevent the clogging-up of chromatography columns. The texture of the protein extract was viscous, suggesting the possible presence of DNA. Therefore, protamine sulphate (0.14 %, w/v) was added to crude tissue extracts to precipitate nucleic acids, prior to ammonium sulphate precipitation (figure 2.3). This process enabled the retention of most of the DCA-*N*-MT activity present in crude root extract, thus allowing seven times more DCA-*N*-MT to be loaded onto the DEAE-Sepharose column than in procedure 1 (compare tables 4.3 and 4.4).

The extract obtained was applied to the chromatographic column sequence described in procedure 1, using linear elution gradients. As illustrated by figure 4.14, this modification improved the purification of DCA-*N*-MT on DEAE-Sepharose with a 20–fold purification in this step alone, as opposed to the six-fold purification obtained for this step in procedure 1 (compare tables 4.3 and 4.4). When applied to the phenyl-

	Total protein (mg)	*SA (nkat.g^{-1})*	*Purification (fold)*	*Yield (%)*
Crude	406.0	11.2	1.0	100.0
Protamine sulphate	469.0	3.2	0.3	33.0
$(NH_4)_2SO_4$	153.0	9.8	0.9	32.9
Desalted	126.3	15.7	1.4	43.6
DEAE-Sepharose	1.2	333.8	29.8	8.8
Resource Q	0.02	4486.4	400.6	1.7

Table 4.4: Balance sheet for the purification of DCA-*N*-MT following procedure 2. Crude extract from 12 day-old soybean roots (570 g) was treated with 0.14 % (w/v) protamine sulphate and centrifuged to remove nucleic acids. The supernatant was then treated with 40-70 % saturation ammonium sulphate. The precipitated protein was subsequently desalted using a Sephadex G-25 (PD10) column and was applied to a DEAE-Sepharose column. Active fractions were then loaded onto a Resource Q column. SA: specific activity.

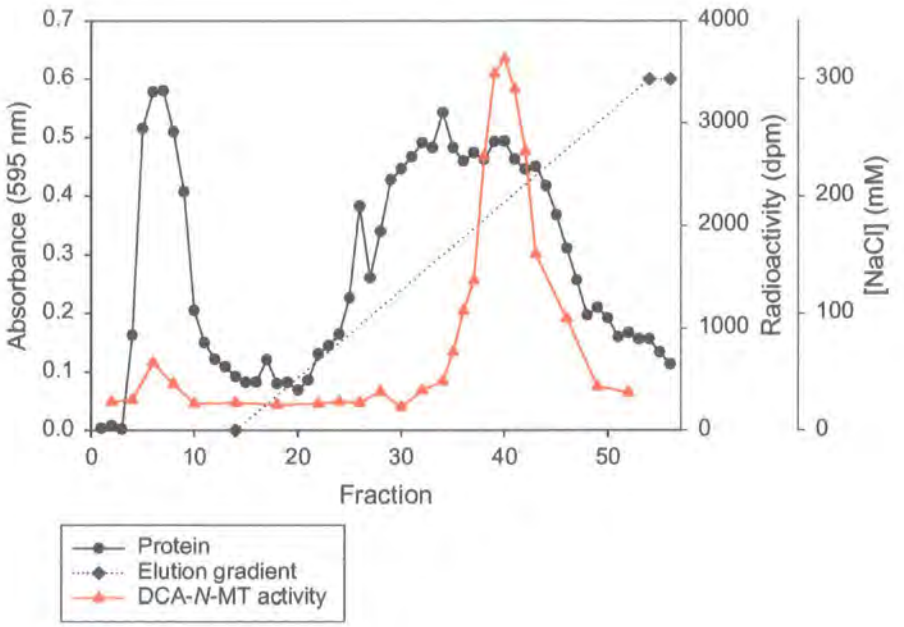


Figure 4.14: Elution profile of DCA-*N*-MT from a DEAE-Sepharose column, using a linear NaCl gradient.

Desalted protein extract was applied to the column in start buffer (20 mM Tris-HCl, pH = 7.5, 2 mM $MgCl_2$). Using the same buffer, a linear elution gradient between 0-300 mM NaCl was applied at 1.5 ml min^{-1} and 1 ml fractions were collected. The protein content in each fraction was determined using Bradford assay (section 2.4.4) and DCA-*N*-MT assay was performed as described in section 2.3.8.3.

Sepharose and to the Cibacron blue F3G-A columns, linear elution gradients did not give reproducible results (data not shown), their use was therefore discontinued. Considering the efficiency of a linear elution gradient combined with the DEAE-Sepharose chromatography in purifying DCA-*N*-MT, this step was retained and additional columns were tested, in order to achieve higher levels of purification.

4.2.2.3.2. Additional steps

A second ion exchange chromatography, consisting of a quaternary ammonium functional group covalently bound to a polystyrene and divinylbenzene cross-linked matrix was tested, following the DEAE-Sepharose step. As opposed to DEAE-Sepharose, which is a weak anion exchanger, Resource Q is a strong anion exchanger (i.e. permanently ionised) and as such, may retain proteins in a different manner to DEAE and thus help to purify DCA-*N*-MT further. Using a linear NaCl gradient, the peak of DCA-*N*-MT activity was eluted with 200 mM NaCl (figure 4.15). This second ion exchanger afforded a ten-fold purification of DCA-*N*-MT in this step but with a lower enzyme recovery. This was nevertheless utilised as starting material for a size exclusion step using an FPLC Sephadex 75 column. Unfortunately, most proteins eluted as a major broad peak, which did not allow any further purification of DCA-*N*-MT (data not shown).

In order to obtain native DCA-*N*-MT size information without excessive loss of protein, an alternative strategy was employed: active fractions from the DEAE-Sepharose column were directly applied to a Sephacryl S-100 gel filtration column. This modified purification protocol was designated procedure 2.2 and showed that DCA-*N*-MT was eluted with an apparent native molecular weight of 52 ± 2 kDa (figure 4.16).

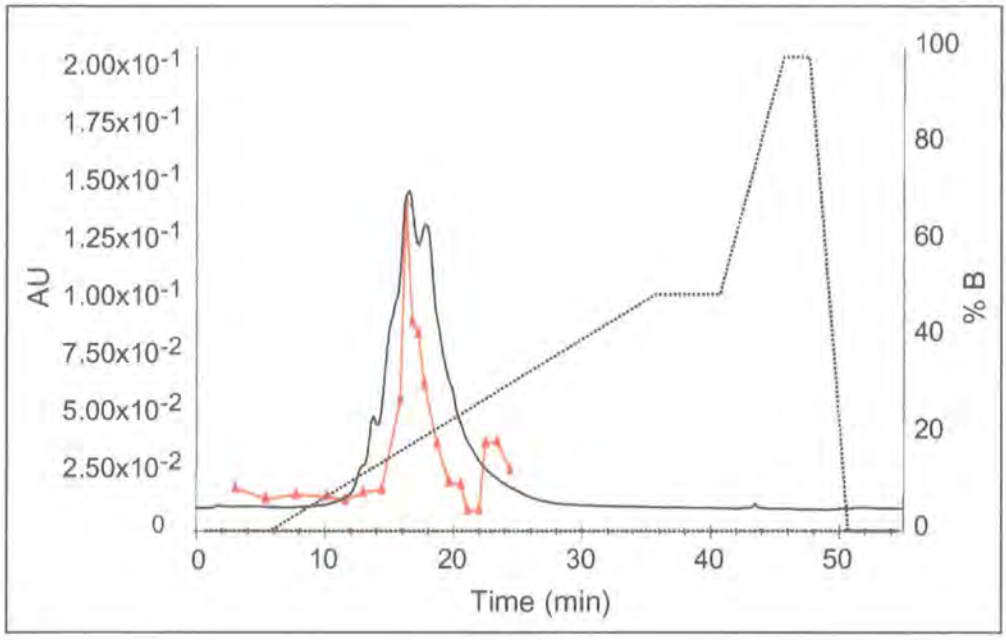
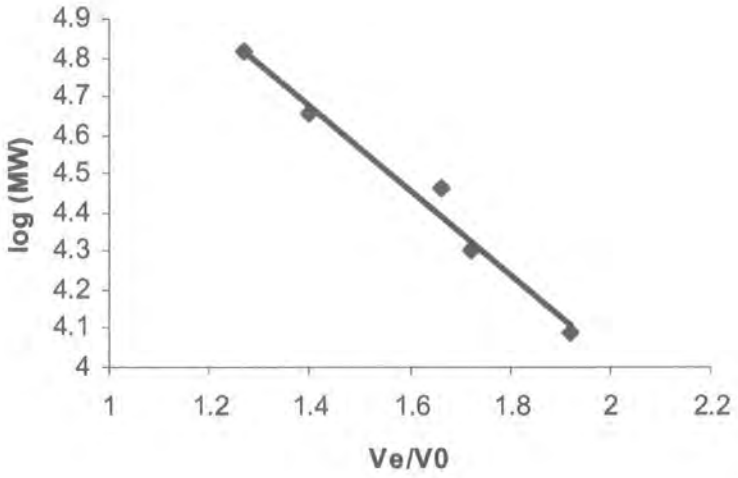


Figure 4.15: Elution profile of DCA-*N*-MT from a Resource Q column.

Proteins were loaded in buffer A (20 mM Tris-HCl, pH = 7.5, 2 mM $MgCl_2$) and eluted with buffer B (1 M NaCl in buffer A). Flow rate: 3 ml min^{-1}. 1.5 ml fractions were collected. DCA-*N*-MT activity was assayed as described in section 2.3.8.3. Dotted line: elution gradient; continuous line: protein; ▲: DCA-*N*-MT activity. AU: arbitrary unit of protein eluted.

A.



B.

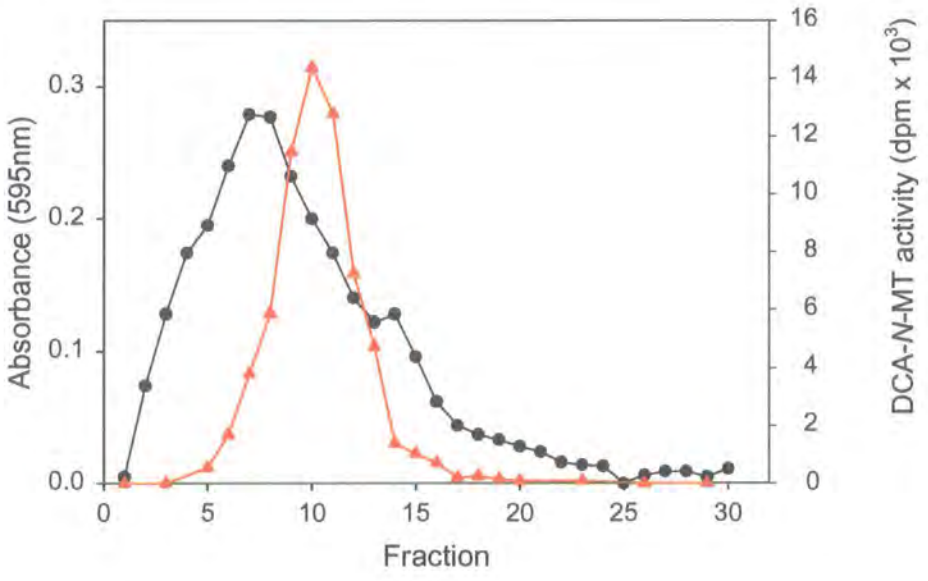


Figure 4.16: Elution profile of DCA-*N*-MT from a Sephacryl S-100 column.

A. Calibration curve for Sephacryl S-100 column, as described in section 2.4.3, step 5''. For DCA-*N*-MT, Ve/V0 ≈ 1.37, which corresponds to a protein with an apparent molecular weight of approximately 52,000. **B.** Active fractions from the DEAE-Sepharose chromatography were applied to a Sephacryl S-100 column in 10 mM Tris-HCl, pH = 8.1, 2 mM $MgCl_2$. Elution rate was 0.5 ml min^{-1} and 1 ml fractions were collected. The protein content in each fraction was determined using Bradford assay (section 2.4.4) and DCA-*N*-MT assay was performed as described in section 2.3.8.3, for 30 min.

4.2.2.3.3. Assessment of purification procedure 2

The balance sheets for the purification of DCA-*N*-MT from 12 d-old soybean roots following procedures 2 and 2.2 (570 g and 160 g of roots respectively) are given in tables 4.4 and 4.5. In both cases, the protein concentration of the protamine sulphate-treated extract was found to be consistently higher than that expected, suggesting the interaction of protamine sulphate with the Bradford assay. The specific activity of DCA-*N*-MT determined in the same extract was repeatedly found to be lower than that in the crude extract, possibly due to the effect of the lower pH of the extract on the enzyme assay (protamine sulphate is dissolved in concentrated HCl before further dilution). Despite this, the utilisation of protamine sulphate in place of Centricon filters allowed a much higher overall yield than that obtained previously. After the ammonium sulphate precipitation, the desalted extract was applied to a DEAE-Sepharose column, from which an overall 30-fold purification was achieved.

In procedure 2, this was followed with a Resource Q column, which added a further 10-fold purification. Consequently, this second scheme led to an overall 400-fold purification of DCA-*N*-MT. To assess this purification visually, active fractions from each step were analysed by SDS-PAGE (figure 4.17). After the Resource Q column, 5 major bands were detected, of 78, 59, 52, 45 and 25 kDa, and were possible candidates for DCA-*N*-MT.

In procedure 2.2, active fractions from the DEAE-Sepharose chromatography were applied directly to a gel filtration column. This step gave a further three-fold purification of DCA-*N*-MT (table 4.5). The purification was also visualised by SDS-PAGE, as illustrated by figure 4.18. Four bands were enriched relative to the ion-exchange column with the 53 kDa band being the most abundant. Since the native size for DCA-*N*-MT was found to be 52 ± 2 kDa on Sephacryl S-100, it is very likely that the 53 kDa band detected by SDS-PAGE corresponded to the enzyme of interest.

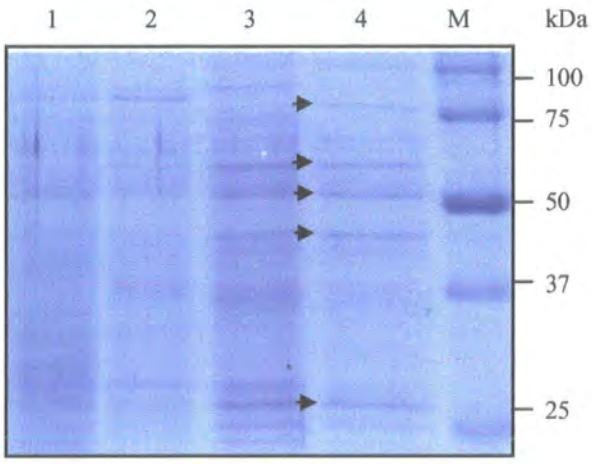


Figure 4.17: SDS-PAGE of DCA-*N*-MT during purification procedure 2.

Samples (4 μg) from active fractions were separated in a 10 % SDS-polyacrylamide slab gel and stained with Coomassie blue. 1, crude extract; 2, desalted precipitate; 3, DEAE-Sepharose; 4, Resource Q; M: molecular weight marker. The arrows indicate the bands that were enriched during the purification. Their apparent size is: 78, 59, 52, 45 and 25 kDa.

	Total protein (mg)	*SA (nkat.g^{-1})*	*Purification (fold)*	*Yield (%)*
Crude	111.7	14.8	1.0	100.0
$(NH_4)_2SO_4$	133.0	34.9	2.4	281.2
Desalted	49.0	26.0	1.8	77.0
DEAE-Sepharose	1.2	476.9	32.2	34.6
Sephacryl S-100	0.04	1382.0	93.4	3.3

Table 4.5: Balance sheet for the purification of DCA-*N*-MT following procedure 2.2.

Crude extract from 12 day-old soybean roots (160 g) was treated with 0.14 % (w/v) protamine sulphate and centrifuged to remove nucleic acids. The supernatant was then treated with 40-70 % saturation ammonium sulphate. The precipitated protein was subsequently desalted using a Sephadex G-25 (PD10) column and was applied to a DEAE-Sepharose column. Active fractions were then applied to a Sephacryl S-100 column. SA: specific activity.

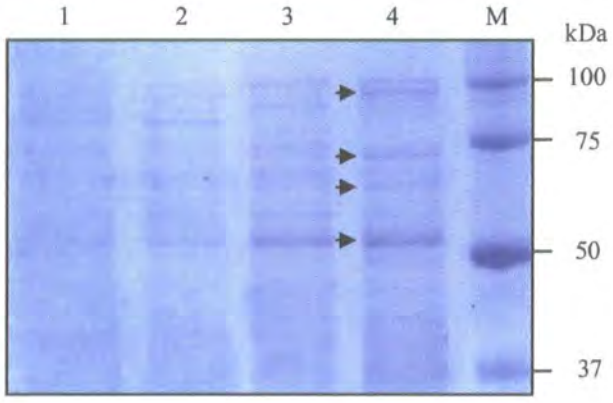


Figure 4.18: SDS-PAGE of DCA-*N*-MT during purification procedure 2.2.

Samples (2 μg) from active fractions were separated in a 10 % SDS-polyacrylamide slab gel and stained with Coomassie blue. Lane 1: crude extract; lane 2: desalted precipitate; lane 3: DEAE-Sepharose; lane 4: Sephacryl S-100; lane M: molecular weight marker. The arrows indicate the bands that were enriched during the purification. Their apparent size is: 92, 73, 66 and 53 kDa.

4.3. Discussion

The aim of this chapter was to purify DCA-*N*-MT from soybean roots. To do so, a specific and sensitive assay for DCA-*N*-MT was developed. Assays for the study of DCA-*N*-MT activity in soybean suspension cultured cells (Sandermann *et al.*, 1991; Schmidt *et al.*, 1995) and in peanut hypocotyls (Matern *et al.*, 1984) were reported. All three assays involved the separation of the reaction products by TLC. The detection and quantification of radiolabelled compounds were rather insensitive, either employing a linear TLC analyser or by scraping off the appropriate regions and quantifying radioactivity by LSC. Here, a simple and sensitive assay, specific for DCA-*N*-MT, was developed. Assay parameters such as time, temperature, pH and buffer were optimised. Moreover, in order to eliminate the need for a TLC analysis followed by the scanning of the plate with a linear analyser, which is a time-consuming and an insensitive detection technique, experiments were carried out to ensure that the reaction products could be directly quantified by LSC (section 4.1). DCA-*N*-MT specific activity in soybean suspension-cultured cells was previously reported to be 32 nkat g^{-1} and from 34.3 to 80.1 nkat g^{-1} (Sandermann *et al.*, 1991 and Schmidt *et al.*, 1995, respectively), whereas a specific activity of 230 nkat g^{-1} was found using the assay presented here (table 3.3). When measured in 13 d-old soybean roots, Schmidt and co-workers (1995) found a DCA-*N*-MT specific activity of 9.6 nkat g^{-1}, as opposed to 73.6 nkat g^{-1} using the present assay (data not shown). These differences could be due to the different soybean variety utilised, which was not indicated by the authors, but it was more likely because of the optimisation of the assay utilised in the present study.

This assay was applied to the purification of DCA-*N*-MT from soybean roots. Although two different strategies were employed, the purification of DCA-*N*-MT to homogeneity was not achieved. When the active fractions were analysed by SDS-PAGE, several protein bands were detected for each purification procedure but only one of them, the 52-53 kDa band, was consistently present (figures 4.13, 4.17 and 4.18). Under native conditions, when active fractions were analysed by gel filtration on a HiPrep Sephacryl S-100 high resolution column, DCA-*N*-MT activity was assigned to a

52 ± 2 kDa molecule (figure 4.16). Therefore, assuming that the enzyme of interest is a monomer, the 52-53 kDa band observed after SDS-PAGE may indeed correspond to DCA-*N*-MT. According to the literature, only two DCA-*N*-MT have been purified but neither to homogeneity. DCA-*N*-MT was purified 74-fold from soybean cultured cells (Sandermann *et al.*, 1991) and partially from peanut seedlings (the authors did not report the fold purification, Matern *et al.*, 1984). These enzymes were reported to be 48 ± 3 kDa and 45 kDa, respectively, as determined by gel filtration. In the case of the DCA-*N*-MT from soybean, a protein band at 48 kDa was also detected after SDS-PAGE, suggesting that the enzyme is a monomer (Sandermann *et al.*, 1991). Since it is not clear to which family DCA-*N*-MT belongs and some *N*-MT have been found to accept several substrates (Guo *et al.*, 1993; Wu *et al.*, 1995), it is appropriate to consider published data regarding other *N*-MTs. 1-aminocyclopropane-1-carboxylate *N*-malonyltransferases (ACC-*N*-MT) were purified from mung bean and tomato fruit (Guo *et al.*, 1992; Martin and Saftner, 1995) and D-amino acid *N*-MTs from peanut, mung bean and tomato leaves (Matern *et al.*, 1984; Guo *et al.*, 1993 and Wu *et al.*, 1995). The size of the purified *N*-MTs ranged from 38 kDa to 55 kDa, as determined by gel filtration. But most importantly, these proteins had a similar apparent size when analysed by SDS-PAGE, suggesting that *N*-MTs are all monomers.

4.4. Summary

Although attempts to purify DCA-*N*-MT have been previously reported, the enzyme was purified only 70-fold from soybean suspension-cultured cells (Sandermann *et al.*, 1991). Moreover, the reaction assays employed were tedious and lacked precision. Here a simple, robust and sensitive radiometric assay specific for DCA-*N*-MT was developed. Using this assay, DCA-*N*-MT was purified 400-fold from soybean roots following protamine sulphate and ammonium sulphate precipitations and two ion exchange chromatographies, namely DEAE-Sepharose and Resource Q columns. DCA-*N*-MT activity was assigned to a 52 ± 2 kDa protein based on results obtained from gel filtration and SDS-PAGE. DCA-*N*-MT was investigated further in chapter 5, not only to

confirm that the 52 ± 2 kDa protein corresponds to DCA-*N*-MT but also to find an alternative means of obtaining peptide sequence information.

Chapter 5

Further characterisation of DCA-*N*-MT: towards a molecular identification

5.1. Introduction

Following the identification of *N*-malonylation as a major route of DCA detoxification in soybean, a major aim of this thesis was to identify and clone a cDNA encoding DCA-*N*-MT. Since no *N*-malonyltransferases have been cloned to date, the original strategy adopted was a classical approach to protein sequence identification via protein purification, which was described in Chapter 4. Purification to near-homogeneity proved difficult during the time-scale of the project, therefore alternative methods to identify the DCA-*N*-MT protein and cDNA were sought.

The first alternative strategy considered was a candidate gene approach, i.e. to identify and isolate DCA-*N*-MT sequences using sequence information from genes encoding enzymes of detoxification and secondary metabolism. To date, no sequence information regarding xenobiotic-specific or other *N*-malonyltransferases has been published, but the cloning of three *O*-malonyltransferases involved in the biosynthesis of anthocyanins has been reported recently (Suzuki *et al.*, 2001; 2002). These malonyltransferases were found to belong to the acyltransferase superfamily (St Pierre *et al.*, 1998), which previously did not include transferase enzymes capable of utilising malonyl-CoA as a co-substrate. Although all the family members identified to date

catalyse transfer from CoA esters to hydroxyl and not amine groups, the recent cloning of DCA-*N*-GT from *Arabidopsis* and its identification as a member of the *O*-GT superfamily (Loutre *et al.*, 2003) suggest that it is conceivable that DCA-*N*-MT is a member of the *O*-acyltransferase superfamily. When the protein sequences of *O*-MTs were aligned with those of other members of the acyltransferase family, it was deemed possible to design PCR primers to motifs conserved in anthocyanin-metabolising *O*-MTs which could be used to amplify homologous *O*-MTs from soybean (data not shown). However, this would most likely result in the isolation of partial clones representing anthocyanin-metabolising *O*-MTs and not DCA-*N*-MT. Since it is not possible to predict either the substrate or the type of acyl donor based on the primary sequences of *O*-acetyltransferase proteins, it was concluded that isolation of soybean *O*-MT cDNAs as a potential route to identifying DCA-*N*-MT was too risky a strategy to adopt.

Since a specific and sensitive assay was available to detect DCA-*N*-MT activity, methods of identifying DCA-*N*-MT proteins and/or cDNAs based on the function and the induction of the enzyme were considered. The induction of xenobiotic detoxifying enzymes by herbicides and safeners has been reported widely (Hoagland and Zablotowicz, 2001). Whilst enzymes and transporters of the first three phases of xenobiotic detoxification have been shown to be induced by xenobiotic treatments, the induction of conjugating enzymes has perhaps been best studied (Gaillard *et al.*, 1994; Fraissinet-Tachet *et al.*, 1998; Robineau *et al.*, 1998; Lee and Raskin, 1999). For example, the levels of glutathione *S*-transferase (GST) activity (and also of the co-substrate, glutathione) were shown to be induced by various herbicides and safeners (Hunaiti and Ali, 1991; Alla, 1995; Uotila *et al.*, 1995; Alla and Hassan, 1998). In many cases, the increase in enzyme activity was shown by Northern analysis to be via the induction of GST transcripts (Riechers *et al.*, 1998). The possible induction of DCA-*N*-MT by DCA treatments is interesting since it would lead to potential routes for gene identification. If DCA induces DCA-*N*-MT activity by increasing the level of protein synthesis, the comparison of the protein profiles of treated and untreated samples would potentially identify bands representing differentially expressed proteins. The parallel purification of DCA-treated and control soybean roots would therefore be a means to

identify the bands corresponding to DCA-*N*-MT. Methods based on the induction of mRNA synthesis, such as subtractive cloning (Sagerström *et al.*, 1997), differential screening or differential display (Liang and Pardee, 1992), are also potential routes to identify the gene of interest. However, since the induction of protein synthesis can occur via the activation of either transcriptional or translational mechanisms, a method for obtaining gene information based solely on enzymatic function was desirable. The feasibility of *in vitro* expression cloning (IVEC) was therefore assessed. The principle of this method is to express proteins from a population of cDNAs and to assay for specific biological activities. The cDNA pools are then subdivided in order to identify clone(s) encoding the enzyme of interest (King *et al.*, 1997).

In this chapter, the effect of the model xenobiotic DCA on DCA-*N*-MT extracted from soybean roots and cell cultures was investigated and kinetic studies were performed. From the results obtained, two approaches to identify DCA-*N*-MT proteins and/or cDNAs were taken: the differential enzymatic activity between DCA-treated and control samples were applied firstly to assist expression cloning and secondly to assist conventional purification.

5.2. Effect of DCA on DCA-*N*-MT activity

5.2.1. Suspension-cultured cells

Since DCA is phytotoxic, the effects of DCA pre-treatment on cell viability were tested, to ensure that the dose applied would not be damaging or lethal. Therefore, suspension-cultured soybean cells were grown in the presence of 6.2, 31, 62 and 310 μM DCA and analysed for viability using phenosafranin, which is excluded by viable cells (Li *et al.*, 1999). This experiment demonstrated that cell growth was not affected by 62 μM DCA, but at 310 μM, DCA treatment was lethal (also characterised by the browning of the cells; data not shown). Therefore, a time-course study of DCA-*N*-MT activity after treatment with 50 μM DCA was performed. Since the comparison of the effects of DCA-treatments on soybean suspension-cultured cells and soybean roots would be interesting and because whole organs are generally more robust than cells in

culture, 100 µM DCA treatments were also included. DCA-*N*-MT activity was assayed every 12 h during 36 h, using crude protein extracts. As illustrated by figure 5.1, DCA-*N*-MT activity was found to be constitutively high in suspension-cultured cells, with an average activity of 332.9 ± 38.9 nkat.g^{-1}, and no statistically significant increase was observed in response to DCA pre-treatment.

5.2.2. Plants

Hydroponically-grown soybean seedlings were treated with 100 µM DCA and DCA-*N*-MT was assayed using crude protein extracts. The DCA-*N*-MT activity obtained was compared to that of controls, which were treated with methanol only. Preliminary experiments showed that an increase in DCA-*N*-MT activity in the roots was detectable after 8 h and augmented further after 24 h (results not shown). Following these observations, a more detailed experiment was performed, in which soybean plants were treated with 100 µM and 200 µM DCA. DCA-*N*-MT activity in crude root extracts was assessed every 12 h, over a 48 h period (Figure 5.2.A). Compared to the controls, DCA-*N*-MT activity in DCA-treated plants increased up to three-fold and no further increase was detected after 24 h. The effects of 100 µM and 200 µM DCA were not significantly different. Consequently, 100 µM DCA-treatments were applied thereafter. A shorter time-course experiment (figure 5.2.B) confirmed that a significant increase in activity was only detectable after 6-12 h of DCA pre-treatment.

5.3. Kinetic studies

Earlier experiments on untreated tissue (see section 3.2.5) revealed that the constitutive activity of DCA-*N*-MT was higher in soybean suspension-cultured cells than in roots, suggesting important differences between these tissues. Moreover, DCA treatments of soybean tissues was found to increase the activity of the detoxification enzyme, DCA-*N*-MT, in roots but not in suspension-cultured cells (see above). Kinetic studies of the effect of DCA treatments on both soybean roots and cell cultures might shed some light on the properties of DCA-*N*-MT isoenzymes in the two tissues and also

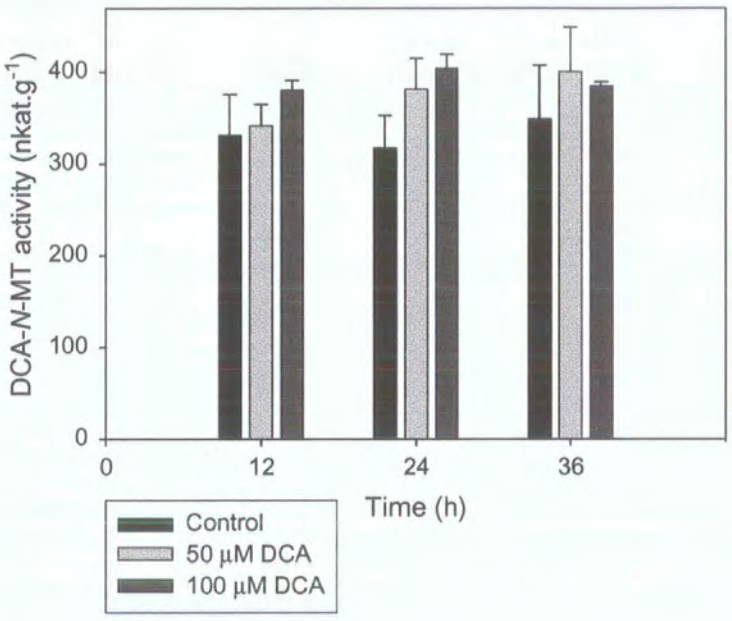


Figure 5.1: Time-course study of DCA-*N*-MT activity in suspension-cultured soybean cells, following DCA treatments.

8 d-old cells were treated with 50 and 100 µM DCA prior to the determination of DCA-*N*-MT activity. Methanol was used as a control. After different treatment times, DCA-*N*-MT activity was assessed in the 10 d-old cell crude extracts. Values given are the mean ± standard deviation of two replicates. Results representative of two experiments.

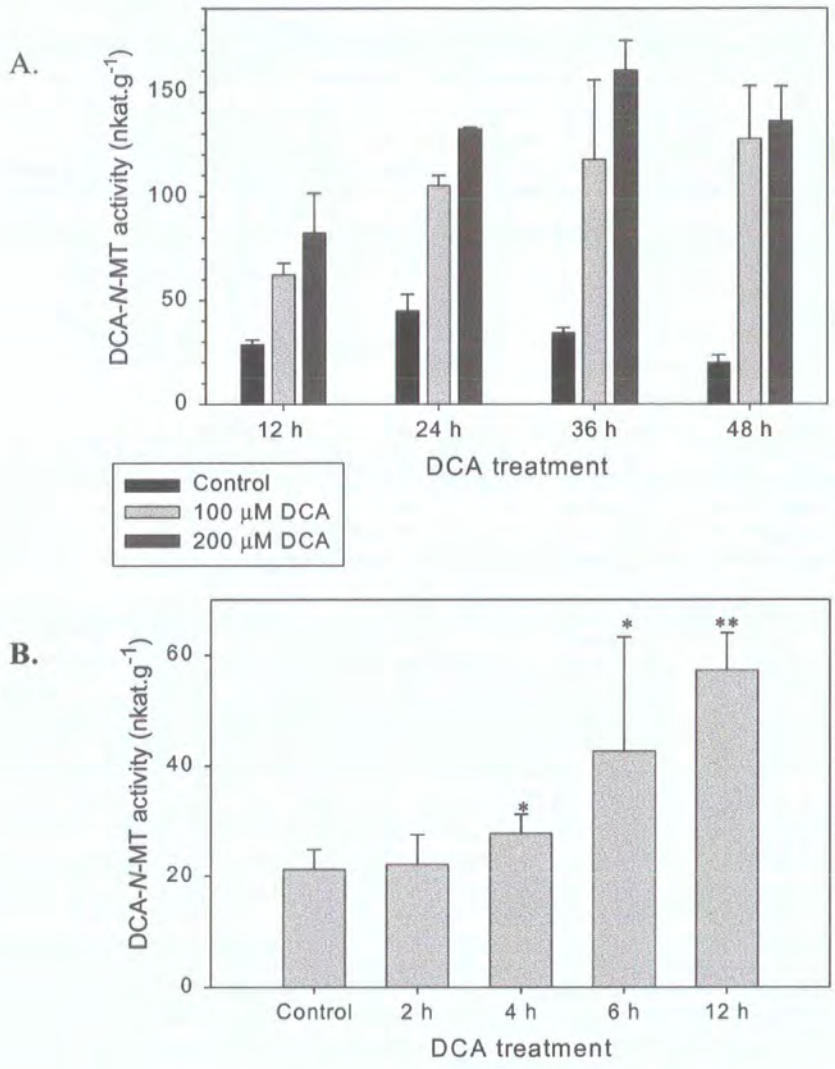


Figure 5.2: Time-course study of DCA-*N*-MT activity in soybean roots, following DCA treatments.

Soybean seedlings were grown hydroponically and treated with various concentrations of DCA prior to the determination of DCA-*N*-MT activity. Methanol was used as a negative control. After different treatment times, DCA-*N*-MT activity was assessed in 10 d-old plant crude extracts, as described in section 2.3.8.3. Values given are the mean ± standard deviation of two replicates. **A**: 100 µM and 200 µM DCA treatments. Results representative of two experiments. **B**: 100 µM DCA treatment. DCA-*N*-MT activity after DCA treatment was significantly different from the control after 4 h (*: $p<0.05$; **: $p<0.005$). Each time-point was done in quadruplicate. Results representative of two experiments.

on the mechanism of induction, for example: whether DCA induction of DCA-*N*-MT activity is due to a change in the enzyme affinity for its substrate(s), to a higher amount of the enzyme itself, or to other factors, such as the induction of additional isoforms. Moreover, K_m values for DCA and malonyl-CoA (M-CoA) would be useful for the optimisation of DCA-*N*-MT assay.

For these studies, protein extracts of soybean tissues were prepared by ammonium sulphate precipitation followed by desalting on a PD10 column. To determine the kinetic constant for DCA-*N*-MT activity towards DCA, a mixture of radiolabelled and cold M-CoA was used and kept at a constant concentration of 1 mM and DCA concentration was varied from 0 to 600 µM. To determine the K_m value for M-CoA, DCA concentration was kept constant at 300 µM (10-fold K_m value for DCA, see below) and the concentration of M-CoA (cold and radiolabelled mixture) was varied from 0 to 1.2 mM. Unfortunately, attempts to determine the K_m value for M-CoA were unsuccessful (data not shown), possibly suggesting that an ammonium sulphate cut is too crude an extract for kinetic studies of a compound such as malonyl-CoA, which is the substrate of many enzymes. Consequently, only the results regarding K_{mDCA} are presented below.

According to Henderson (1992), "there is only one statistically sound method for the determination of the best fit K_m, V_{max} values by visual inspection of a graph. This is the direct linear plot". More traditional plots such as the Lineweaver-Burke plot (1/V against 1/S), the Eadie-Hofstee plot (V against V/S) or the Hanes plot (S/V against S) should not be used for any other purpose than display. Here, these plots were used to ensure that the scatter of data was linear. A direct linear plot was then obtained, in which the coordinates of each intersection provided estimates of K_m and V_{max} with the median values being the best-fit values K_m and V_{max} (Cornish-Bowden and Eisenthal, 1974). The intersection points were ranked from left to right to obtain the K_m value and from bottom to top for V_{max}.

The affinity of DCA-*N*-MT for DCA was determined using enzyme extracts obtained from soybean roots (control and DCA-treated prior to protein extraction). A plot of DCA-*N*-MT activity as a function of DCA concentration is shown in figure 5.3.A. The results demonstrated that (1) the enzyme activity was induced in DCA-

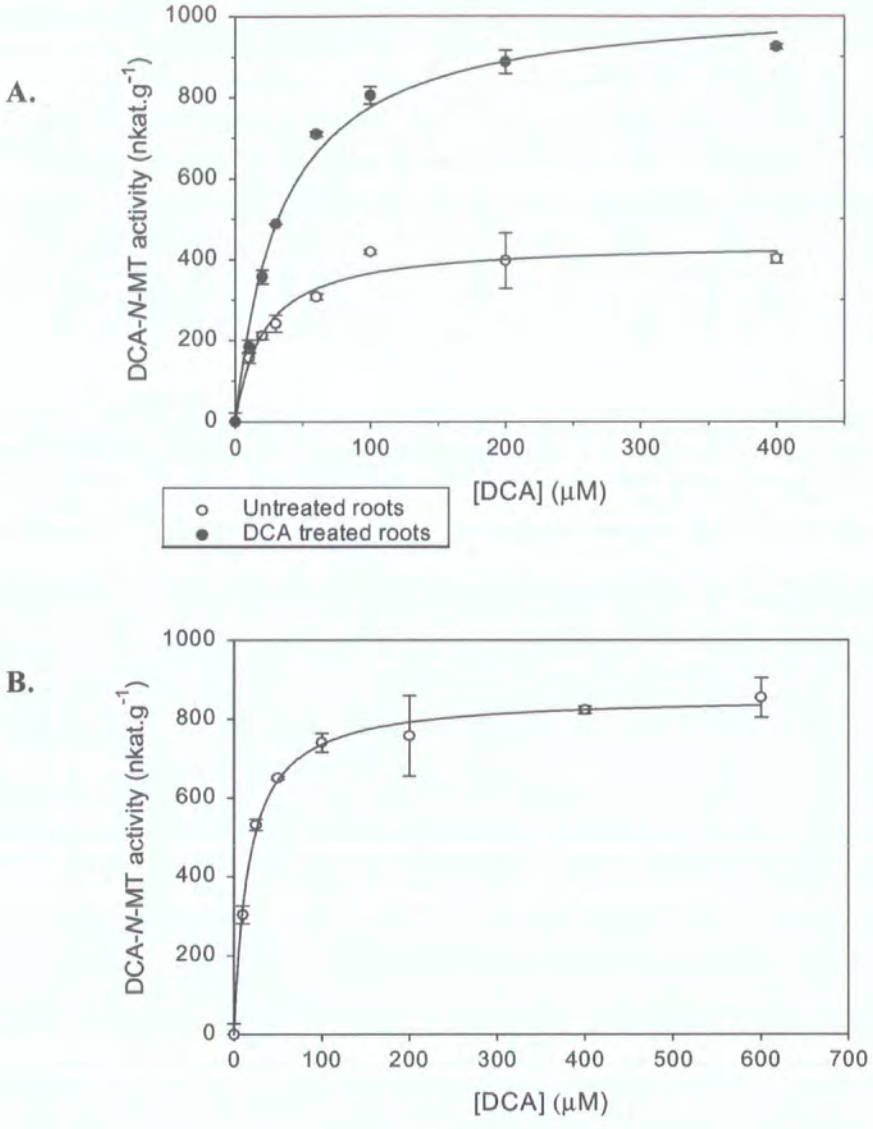


Figure 5.3: Kinetic studies of DCA-*N*-MT activity in soybean tissues.

DCA-*N*-MT activity in various soybean tissues was assessed as described in section 2.3.8.3. except that here, the concentration of DCA was varied. Crude protein extracts were utilised. **A**: 10 d-old soybean roots treated with 100 µM DCA, or methanol (control) for 24 h, prior to protein extraction. **B**: 11 d-old suspension-cultured soybean cells (middle of exponential growth phase). Curves were generated by a least-square fit to a Michaelis and Menten hyperbola (calculated by SigmaPlot 2000). Values given are the mean ± standard deviation of two replicates. Results representative of two experiments.

treated roots, which is consistent with the data obtained previously (figure 5.2) and (2) DCA-*N*-MT obeys Michaelis-Menten kinetics (figure 5.3.A). Using the various plots described above, values of K_m and V_{max} for control roots were found to be K_{mDCA} = 19.5 ± 0.7 μM and V_{max} = 238.8 ± 256.3 nkat.g^{-1} (n = 2). After 100 μM DCA-treatment for 24 h, K_{mDCA} in the roots was 32.0 ± 2.8 μM and V_{max} = 1430.0 ± 565.7 nkat.g^{-1} (n = 2). The K_m value for DCA in suspension-cultured cells was determined as described above, using cells in the middle of the exponential growth phase. From a direct linear plot, values for K_m and V_{max} were found to be 15 μM and 840 nkat.g^{-1}, respectively.

5.4. Mechanism of induction of DCA-*N*-MT activity

The time-course experiments and the kinetic studies shown above suggested that the induction of DCA-*N*-MT activity in soybean roots might be due to *de novo* protein synthesis, since V_{max} should be proportional to the number of active sites. Investigation of the mechanism by which this induction occurred would be important to determine which strategy to pursue for the identification of DCA-*N*-MT. Inhibitors of transcription and translation were therefore employed to determine whether either or both of these processes were required for the induction of DCA-*N*-MT activity.

5.4.1. Effects of inhibitors

To determine the stage at which DCA-*N*-MT activity was induced, the effects of common RNA and protein synthesis inhibitors on enzyme activity were assayed following DCA pre-treatment of tissue. Actinomycin D (actD), an antibiotic from *Streptomyces*, binds tightly to double-stranded DNA, thereby preventing its association with the transcription machinery and inhibiting transcription. Cycloheximide (CH) inhibits the peptidyl transferase activity of the 60S ribosomal sub-unit, thus blocking polypeptide chain formation, and consequently inhibiting translation. As shown in figure 5.2.A, DCA treatments for periods longer than 24 h did not result in a further, significant increase in DCA-*N*-MT activity. Therefore, fresh soybean plants were treated for 24 h with DCA (100 μM), actD (100 μg ml^{-1}) and CH (10 μg ml^{-1}), either

alone or as combinations of DCA with inhibitor. Even though three plants were used for each treatment (each in duplicate), the results of three independent experiments were inconclusive (data not shown). After 24 h incubation with either actD or CH, treated plants did not look as healthy as the control plants, suggesting that exposure to the inhibitors was toxic. It was thus necessary to shorten the length of the treatment. Unfortunately, since a 12 h DCA pre-treatment was necessary to ensure the significant induction of DCA (figure 5.2), the utilisation of RNA and protein synthesis inhibitors was abandoned and an alternative method to determine the stage at which DCA induces DCA-*N*-MT activity was applied.

5.4.2. *In vitro* translation

Since the mechanism by which DCA was inducing DCA-*N*-MT activity was not determined unequivocally, the use of differential cloning techniques to clone DCA-*N*-MT was not attempted. The availability of a simple and specific assay for DCA-*N*-MT suggested that *in vitro* expression cloning (IVEC) would be a possible method to isolate cDNAs encoding DCA-*N*-MT. IVEC exploits the biological activity of an enzyme to identify the cDNA from which the polypeptide originated. Basically, the method consists of constructing a cDNA library in a plasmid containing a T7 or T3 RNA polymerase promoter, which is subdivided into pools and subsequently transcribed and translated *in vitro* (e.g. using the TNT® Coupled Reticulocyte Lysate System manufactured by Promega; King *et al.*, 1997). The resulting proteins are then screened for enzymatic activity to determine the clone(s) containing the gene of interest. An alternative method is to express protein from a conventional cDNA library but this method has the disadvantage that fewer clones will yield functional protein (Thomas *et al.*, 1999).

To test whether the application of IVEC to this project was feasible, total RNAs extracted from soybean tissues were translated *in vitro* using a Rabbit Reticulocyte Lysate System (RRL, see section 2.5.4.2), and the translation products were assayed for DCA-*N*-MT activity. Total RNAs extracted from soybean roots (± DCA treatment) and cell cultures were used as starting material for *in vitro* translation using RRL. The DCA-

N-MT assay was performed as described in section 2.3.8.3, except that approximately 0.9 mg protein was used per assay, with 31 µM M-CoA (consisting of a mixture of 9 µM radiolabelled and 22 µM cold M-CoA) and an incubation time of 1 h 30 min was employed. A significant difference in DCA-*N*-MT activity between the water control and the samples containing RNA was observed, suggesting that the *in vitro* translation of DCA-*N*-MT from background of other RNA species was successful (figure 5.4). However, the apparent background DCA-*N*-MT activity in the control was very high and no significant difference was detected between DCA-treated and untreated root samples, nor between root and cell samples, even though DCA-treated roots and suspension-cultured cells possess more than twice the DCA-*N*-MT activity in roots (section 5.2). Due to time constraints, this approach was not investigated further.

5.5. Identification of proteins induced by DCA pre-treatment

According to the results reported above, DCA-*N*-MT activity increased with pre-treatment of soybean plants with 100 µM DCA. Subsequently, kinetic studies showed that K_{mDCA} did not vary greatly following DCA treatment while V_{max} increased more than three-fold, suggesting possible *de novo* protein synthesis (section 5.3) of either DCA-*N*-MT or of other proteins which could influence its activity, such as enzymes leading to the formation of co-substrates or as yet unknown activation factors. Since a partial purification of DCA-*N*-MT had been established in Chapter 4, it was decided to test whether the two to three-fold increase in DCA-*N*-MT activity following DCA pre-treatment was reflected in the increased abundance of specific proteins in the partially-purified preparation, using either one-dimensional (1D) SDS-PAGE or two-dimensional (2D) differential gel electrophoresis (DiGE). It was hoped that this would permit the confident identification of protein bands or spots corresponding to DCA-*N*-MT, which would be a starting point for the molecular characterisation of the enzyme. Consequently, the purification of DCA-*N*-MT from DCA-treated and control soybean roots was carried out.

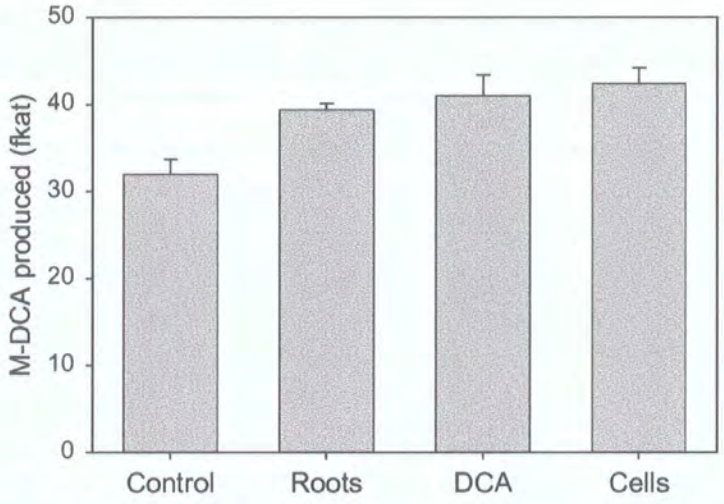


Figure 5.4: DCA-*N*-MT activity in *in vitro* translation samples.

6 µg total RNA, extracted from various soybean tissues, was translated *in vitro* using rabbit reticulocyte lysate (see section 2.5.4). Translation products were then assayed for DCA-*N*-MT activity (as described in section 2.3.8.3 except that the incubation was at 35 °C for 90 min). Values represent the means ± standard deviation of duplicate measurements. Control: water. Roots: RNA extracted from 10 d-old roots. DCA: RNA extracted from 10 d-old roots treated with 100 µM DCA for 24 h. Cells: RNA extracted from 11 d-old suspension-cultured cells.

5.5.1. Purification of DCA-*N*-MT from control and DCA-treated soybean roots

In Chapter 4, purification procedure 2 was found to be a better scheme for DCA-*N*-MT purification than purification procedure 1. However, the FPLC Sephadex 75 column did not purify the enzyme further, therefore it was replaced with a conventional gel filtration column and the second ion-exchange column (Resource Q) was omitted to limit enzyme degradation and to shorten the purification procedure (see section 4.2.2.3.2). Briefly, (1) nucleic acids in crude protein extracts were precipitated with protamine sulphate, (2) proteins in the supernatant were precipitated with ammonium sulphate, (3) the precipitate was re-dissolved and desalted using a PD10 column, (4) protein extract was applied to a DEAE-Sepharose column and (5) a final separation was conducted using a Sephacryl S-100 column. In contrast to the protocol developed in Chapter 4, 5 mM DTT was included in buffers for the later chromatography steps in order to prevent oxidative inhibition of DCA-*N*-MT. Several studies have suggested the presence of sulfhydryl group(s) in the active site of *N*-MT: for example, mersalic acid strongly inhibited ACC-*N*-MT purified from tomato and chickpea (Martin and Saftner, 1995; Marinez-Reina *et al.*, 1996). Also 0.1 mM *p*-chloromercuribenzoate inhibited soybean DCA-*N*-MT but activity could be restored following incubation with DTT (Sandermann *et al.*, 1991). Since loss of activity with time was observed during the purification and assay of DCA-*N*-MT in this study, it was decided to supplement buffers with DTT in order to preserve activity and thus obtain a more realistic estimation of purification.

Following ion exchange chromatography, the peak of DCA-*N*-MT activity in both DCA-treated and control plants was eluted with approximately 200 mM NaCl (figure 5.5), which is consistent with the results obtained in Chapter 4. Note here that DCA-*N*-MT activity in DCA-treated roots was more than two-fold higher than that in the control roots. After the DEAE-Sepharose column, the active fractions were pooled and loaded onto a Sephacryl S-100 column for a size exclusion step. The elution volume for both samples was similar (figure 5.6), and corresponded to that of a 52 ± 2 kDa size protein in both cases. A balance sheet for the purification described above is given in table 5.1. Overall, DCA-*N*-MT was purified approximately 93-fold and 75-fold from the

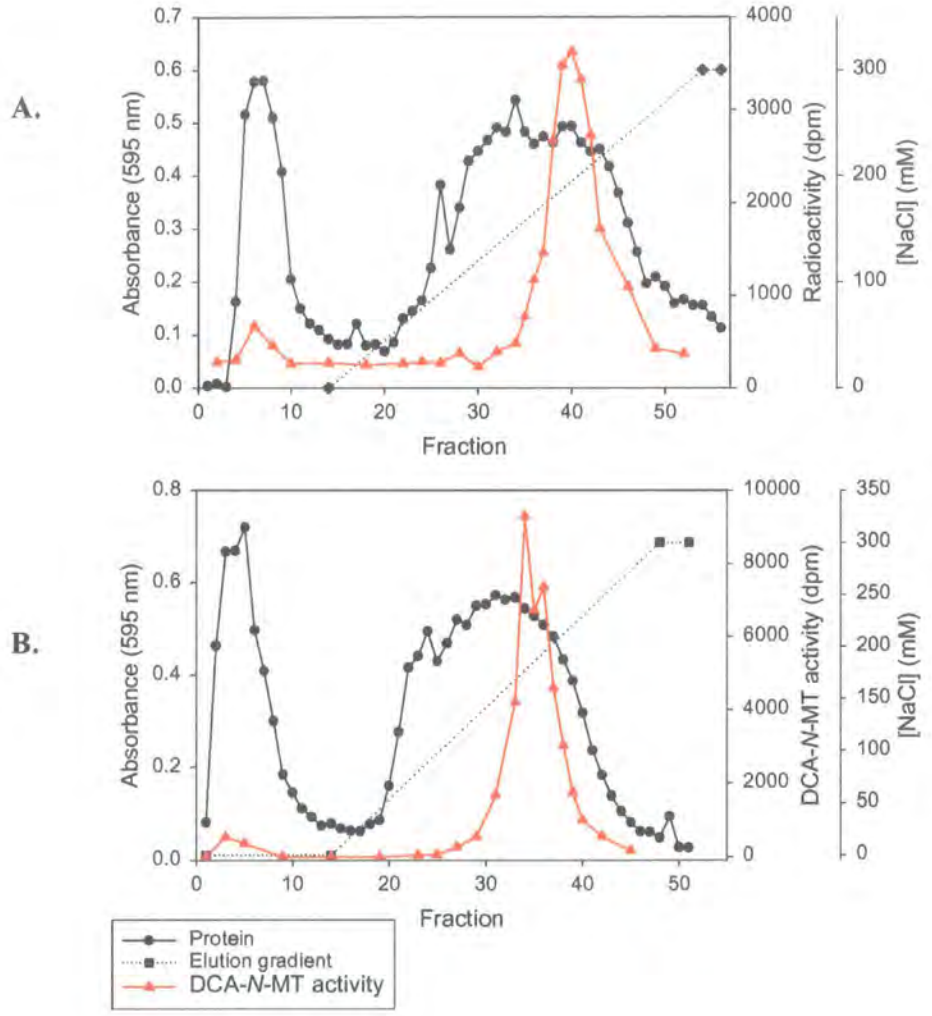


Figure 5.5: Elution profile of DCA-*N*-MT from control and DCA-treated soybean roots, after a DEAE-Sepharose column.

10 d-old soybean seedlings were treated with 100 µM DCA or methanol (control) for 24 h prior to protein extraction. Desalted protein extract was applied to the column in start buffer (20 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$). Using the same buffer, a linear elution gradient between 0-300 mM NaCl was applied at 1.5 ml min^{-1} and 1 ml fractions were collected. The protein content in each fraction was determined using a Bradford assay and DCA-*N*-MT assay was performed as described in section 2.3.8.3. **A:** Control. **B:** DCA-treated seedlings.

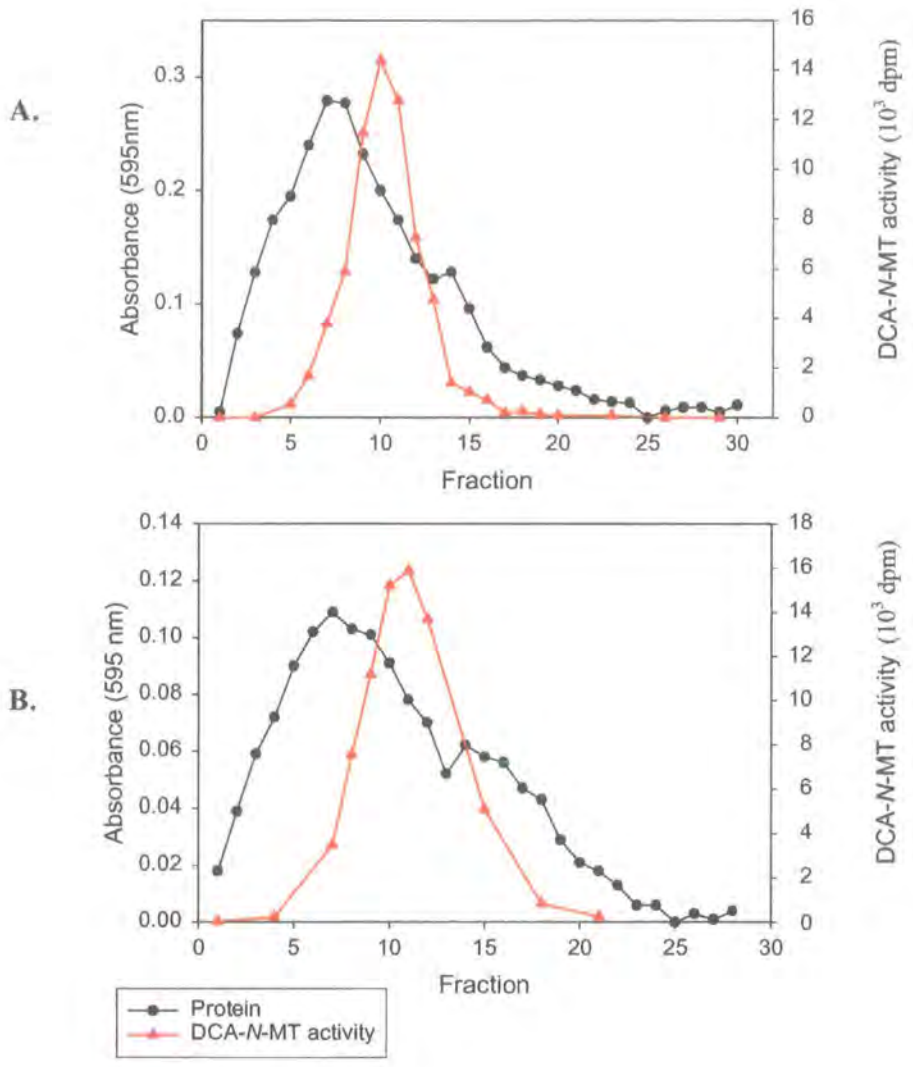


Figure 5.6: Elution profile of DCA-*N*-MT from control and DCA-treated soybean roots, after a Sephacryl S-100 column.

10 d-old soybean seedlings were treated with 100 µM DCA or methanol (control) for 24 h prior to protein extraction. Active fractions from the DEAE-Sepharose column were applied to the column in 10 mM Tris-HCl, pH 8.1, 2 mM $MgCl_2$. Elution rate was 0.5 ml min^{-1} and 1 ml fractions were collected. The protein content in each fraction was determined using the Bradford assay and DCA-*N*-MT assay was performed as described in section 2.3.8.3, for 30 min. **A:** Control. **B:** DCA-treated seedlings.

	Total protein (mg)	*SA (nkat g^{-1})*	***Purification (fold)***	***Yield (%)***
Control-crude	111.7	14.8	1.0	100.0
$(NH_4)_2SO_4$	133.0	34.9	2.4	281.2
Desalted	49.0	26.0	1.8	77.0
DEAE-Sepharose	1.2	476.9	32.2	34.6
Sephacryl S-100	0.04	1382.0	93.4	3.3
DCA-crude	156.9	31.6	1.0	100.0
$(NH_4)_2SO_4$	141.6	35.4	1.1	101.2
Desalted	59.9	45.7	1.4	57.1
DEAE-Sepharose	1.0	760.9	24.1	15.3
Sephacryl S-100	0.07	2360.6	74.7	3.3

Table 5.1: Balance sheet for the purification of DCA-*N*-MT, from control and DCA-treated soybean roots.

10 d-old soybean plants were treated for 24 h with 100 μM DCA or methanol (control) prior to protein extraction. The extracts from control plants (160 g roots) and DCA-treated plants (170 g roots) were mixed with 0.14 % (w/v) protamine sulphate and centrifuged to remove nucleic acids. The supernatant was then treated with 40-70 % saturation ammonium sulphate. The precipitated protein was subsequently desalted using a Sephadex G-25 (PD10) column and was applied to DEAE-Sepharose column. Active fractions were then loaded onto a Sephacryl S-100 column. Data for the methanol control are from table 4.5 in chapter 4. SA: specific activity.

control and DCA-treated roots, respectively, and throughout the purification, DCA-*N*-MT activity in DCA-treated roots was nearly double that in the control roots, reflecting the effect of DCA pre-treatment.

In order to compare the purification of DCA-*N*-MT from control and DCA-treated roots, the active fractions from the crude extract, the desalted precipitate, the ion exchange column and the gel filtration column were analysed by 1D SDS-PAGE (figure 5.7, panels A and B). As shown in figure 5.7, panel C, the intensity of a 53 kDa band was higher in the DCA-treated sample than in the control. Given that this band was identified as a candidate for DCA-*N-MT* in Chapter 4, this observation strongly suggested that DCA-treatment of soybean prior to protein extraction augmented DCA-*N*-MT activity because of *de novo* synthesis of the enzyme itself. However, since SDS-PAGE separates proteins based on their size only, it was very likely that the 53 kDa band corresponded to several different proteins. Therefore, a further step into the purification of DCA-*N*-MT was to use 2D gel electrophoresis, as it separates proteins not only according to their size but also according to their isoelectric point (pI).

5.5.2. Differential gel electrophoresis (DiGE)

An inherent problem regarding the comparison of proteins on different 2D gels is the poor reproducibility of the electrophoretic separation. To circumvent this problem, Ünlü and co-workers (1997) described a method to reproducibly detect differences between two protein samples, by running them in a same gel. To enable the distinction between the two protein samples, equal amounts of each sample are tagged with fluorescent dyes (Cy3 and Cy5) and combined prior to their separation by isoelectric focussing (IEF) and SDS-PAGE. Subsequent fluorescence imaging of the gel at different wavelengths, with the superimposition of the two images thus permits the detection of proteins whose abundance differs in the two samples. This technology has been commercialised by Amersham and was carried out on a service basis by the BBSRC Cambridge Centre for Proteomics.

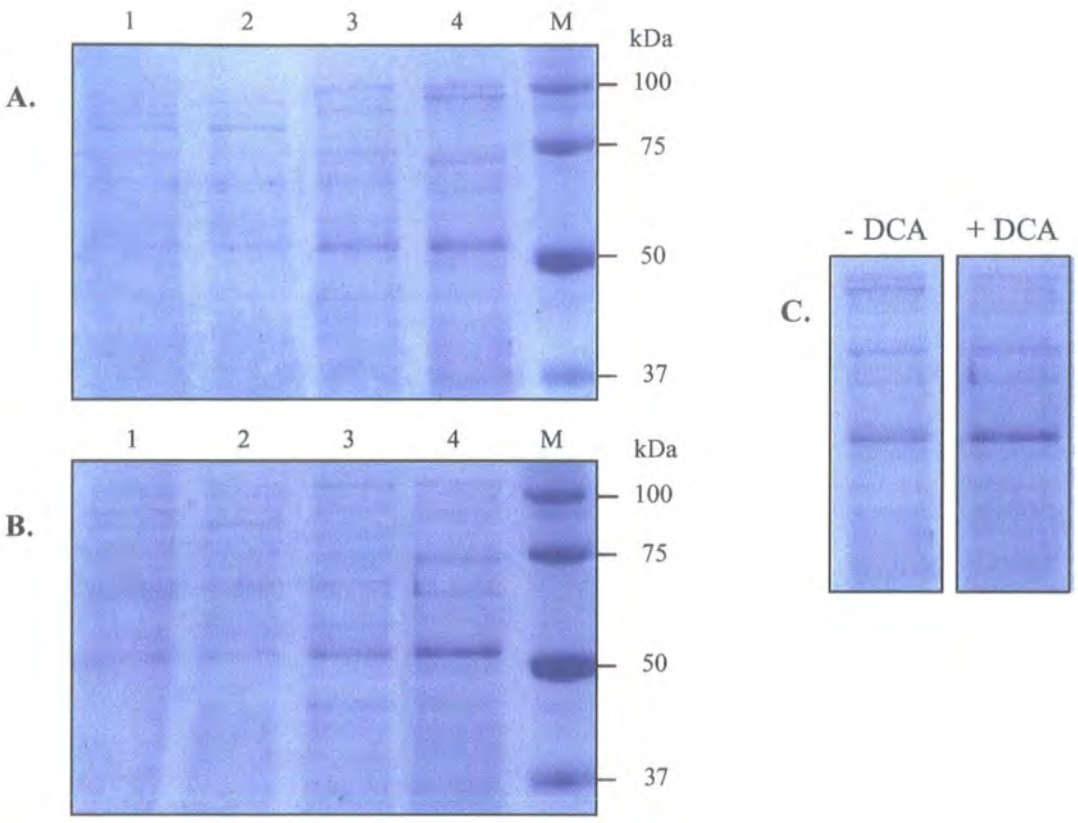


Figure 5.7: SDS-PAGE of DCA-*N*-MT with and without DCA-treatment.

Samples (2 µg) from active fractions were separated in a 10 % SDS-polyacrylamide slab gel and stained with Coomassie blue. Lane 1: crude extract; lane 2: desalted ammonium sulphate precipitate; lane 3: DEAE-Sepharose; lane 4: Sephacryl S-100; lane M: molecular weight marker. **Panel A:** Control (plants exposed to methanol). Reproduced from figure 4.18. **Panel B:** Soybean roots were treated with 100 µM DCA for 24 h prior to DCA-*N*-MT extraction. **Panel C:** Lanes 4 from panels A and B, for a direct comparison of protein profiles with and without DCA-treatment, after gel filtration on Sephacryl S-100.

According to Sandermann and co-workers (1991), DCA-*N*-MT extracted from soybean suspension-cultured cells has a molecular mass of 48 ± 3 kDa and a pI of 6.0. Here, a 52 ± 2 kDa protein was a candidate for DCA-*N*-MT but no information about its pI was available. Therefore, a DiGE analysis was performed using a pH gradient ranging from 4 to 7 in the first dimension and a 12 % acrylamide SDS-PAGE in the second dimension. For each gel, active fractions from the gel filtration step from control and DCA-treated material were labelled with Cy3 and Cy5 respectively, combined and subjected to 2D electrophoresis. Gels were then scanned with the appropriate excitation and emission wavelengths for Cy3 and Cy5 and the images superimposed (figure 5.8, panel A). Where the two dyes were superimposed and proteins were of equal abundance in the two samples, the red and green fluorescent signals corresponding to Cy3 and Cy5 respectively gave rise to a yellow colouration. The green colour represented proteins of higher abundance in the control sample and the red colour corresponded to proteins induced by DCA-treatment. Since size markers are routinely omitted from DiGE experiments in order to prevent protein contamination (K. Lilley, pers. comm.), all spots of differential abundance in the two samples were picked, digested by trypsin and analysed by mass spectrometry. The 2D gel, after silver staining, is shown in figure 5.8 (panel B). The proteins identified are indicated by an arrow. The calculated M_r and fold-induction/repression of hits are shown in table 5.2 and the full results of the MASCOT search are presented in Appendix 2. 37 spots were analysed in total. Of these, 10 corresponded to annotated proteins in the public sequence databases. Several hits corresponded to more than one spot, suggesting the presence of post-translational modifications. Of the identified DCA-induced proteins, disappointingly, none was an obvious candidate for DCA-*N*-MT. Only one protein identified had a calculated molecular weight close to that of DCA-*N*-MT: this was UDP-glucose pyrophosphorylase (52 kDa), which was represented by three spots. The only identified protein with a known role in herbicide metabolism was a glutathione *S*-transferase (GmGST8; two spots), which together with ascorbate peroxidase (one spot) and superoxide dismutase (three spots) is also associated with oxidative stress responses in plants. Interestingly, one protein down-regulated by DCA treatment was identified as phosphoglucomutase.

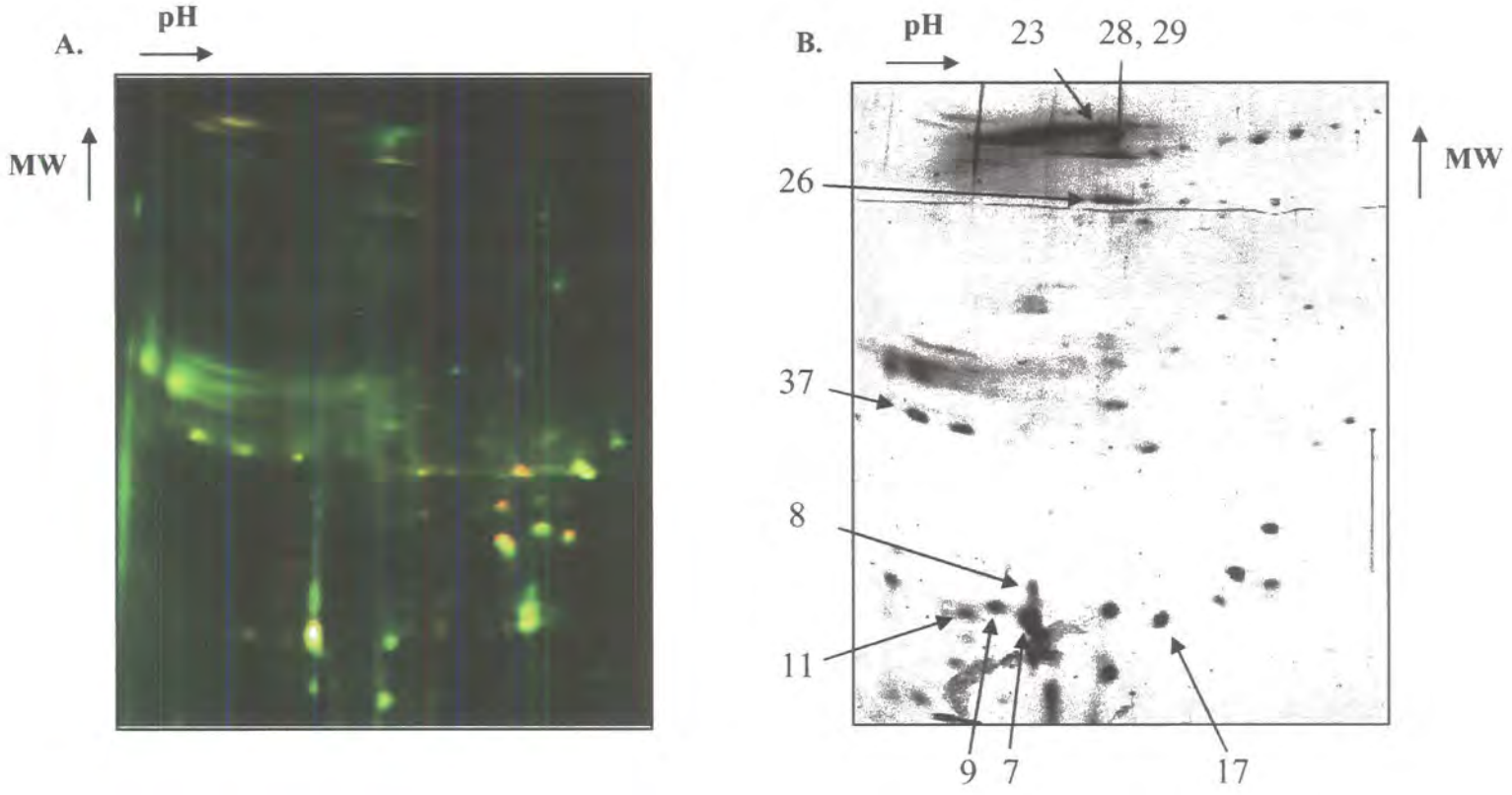


Figure 5.8: DiGE analysis of partially purified DCA-*N*-MT.

Partially purified DCA-*N*-MT from control and DCA-treated material (see section 5.5.2) were labelled with Cy3 and Cy5 respectively, combined and subjected to DiGE analysis (pH 4 to 7 in the first dimension and 12 % acrylamide SDS-PAGE in the second dimension). **Panel A:** Fluorescent image of the gel. Proteins from the control are green, those from the DCA-treated sample are red. Where the two dyes were superimposed, a yellow colour appeared. **Panel B:** Silver stained gel. The spots indicated by the arrows represent proteins whose abundance was regulated by DCA treatment. The identity of these proteins is given in table 5.2.

Spot number	Identity of hit	Accession number	Mass (calc.)	Peptides matched	Total score	Fold change in DCA-treated samples
7	Glutathione-*S*-transferase 8, *Glycine max*	AAG34798	25,893	9	229	+ 2.05 and 1.52
8	Glutathione-*S*-transferase 8, *Glycine max*	AAG34798	25,893	3	95	+ 1.98
9	Superoxide dismutase [Cu-Zn], chloroplast precursor, *Lycopersicon esculentum*	P14831	22,238	1	65	+ 4.45
11	Superoxide dismutase [Cu-Zn], chloroplast precursor, *Lycopersicon esculentum*	P14831	22,328	1	70	+ 2.61
17	Superoxide dismutase [Cu-Zn], chloroplast precursor, *Lycopersicon esculentum*	P14831	22,328	2	59	+ 2.95
23	Phosphoglucomutase, *Tetrahymena thermophila*	AAB97159	65,720	1	53	- 2.23
26	Putative UDP-glucose pyrophosphorylase, *Arabidopsis thaliana* At3g03250	AAK32772	51,847	1	44	+ 2.86
28	Putative UDP-glucose pyrophosphorylase, *Arabidopsis thaliana* At3g03250	AAK32772	51,847	3	69	+ 4.31 and 3.84
29	UDP-glucose pyrophosphorylase, *Amorpha fruticosa*	AAL33919	51,666	6	158	+ 3.08
37	L-ascorbate peroxidase, cytosolic isozyme, *Zea mays*	S49914	27,409	2	92	+ 2.60 and 1.96

Table 5.2: Summary of DiGE analysis of soybean proteins whose abundance is regulated by DCA treatment

Partially purified proteins extracted from untreated and DCA-treated soybean roots (see section 5.5.2) were separated on a two dimensional DiGE system. Differentially expressed proteins were prepared and analysed by mass spectrometry as described in section 2.6.3. + and - indicate an increase and a decrease, respectively, in the intensity of a spot. Scores above 51 indicate identity or extensive homology ($p < 0.05$).

5.6. Discussion

The combined aims of this chapter were to characterise DCA-*N*-MT further and to find a means to identify the gene(s) encoding this enzyme. To do so, a time-course study of the effect of DCA on DCA-*N*-MT activity in soybean plants and cell suspension cultures was performed, followed by kinetic studies of the enzyme. The feasibility of *in vitro* expression cloning was examined and since DCA-treatments increased DCA-*N*-MT activity up to three-fold, comparative purification from treated and untreated material was tested and DCA-inducible proteins were identified using DiGE.

Suspension-cultured cells have been used extensively for xenobiotic metabolism studies because they are generally believed to degrade toxic compounds in a qualitatively similar manner to whole plants (Schneider *et al.*, 1984) but at a more rapid rate. This is partly due to the fact that they often exhibit higher activities of xenobiotic-degrading and secondary metabolic enzymes (J. Little, pers. comm.), and also since suspension cultures lack some of the structural components of whole plants, xenobiotic molecules reach the sites of metabolism more rapidly than in whole plants. Other advantages for the utilisation of cell cultures in pesticide metabolism studies include the ease of protein extraction, the increased purity of metabolite preparation and the fact that they are free from micro-organism contamination. Consequently, protocols to evaluate the metabolic fate of xenobiotics using suspension-cultured cells have been published (Ebing *et al.*, 1984; Harms and Langebartels, 1986). The determination of DCA-*N*-MT activity in soybean plants and cell cultures showed that the enzyme was expressed constitutively and that the specific activity was up to 20-fold higher in extracts from cell cultures compared to those from roots (table 3.3 and section 5.2). In the two tissues, K_{mDCA} was of the same order of magnitude (15 μM in cells and 19.5 μM in untreated roots) but V_{max} was much higher in cell cultures than in untreated roots, thus it seems probable that suspension-cultured cells contain greater amounts of DCA-*N*-MT protein than root cells, but it is also possible that suspension-cultured cells contain a different isoform which is more active. These observations are in agreement with the general belief that suspension-cultured cells metabolise xenobiotics more efficiently than whole

plants (Schneider *et al.*, 1984; Höhl and Barz, 1995; Schmidt *et al.*, 1995) and contain higher levels of the appropriate enzymes.

Since xenobiotic-inducing enzymes have frequently been found to be inducible, the effect of DCA treatment on DCA-*N*-MT activity was investigated. In DCA-treated plants, K_{mDCA} increased less than two-fold (from a mean value of 19.5 to 32 μM; two experiments), suggesting that treatment of plants resulted in a slight decrease in the affinity of DCA-*N*-MT for DCA. However, more experiments are required to determine whether the increase in K_{mDCA} is statistically significant. An increase in K_{mDCA} could be due to the induction of additional isoforms of lower affinity following DCA pre-treatment. V_{maxDCA} was also somewhat variable between experiments but increased significantly in each case. This strongly suggests that DCA induced *de novo* synthesis either of the enzyme itself or of some factors that enhance activity.

In order to assess the possibility of applying *in vitro* expression cloning to this project, *in vitro* translation of RNAs extracted from soybean cells and roots was performed and the protein products assayed for DCA-*N*-MT activity. However, this did not permit the detection of a significant difference in DCA-*N*-MT activity between untreated and DCA-treated root samples or between untreated root and suspension-cultured cell samples. This may indicate that the level of translatable DCA-*N*-MT mRNA is not the only factor determining enzyme activity, especially in induction experiments, but this could not be verified since the experiments with actinomycin D were inconclusive. The lack of significant differences between different samples may also be due to the fact that the protein concentration in RRL was very high (36.9 ± 3.9 mg ml^{-1}) and, by comparison, the amount of DCA-*N*-MT protein translated would be negligible. Since the control consisted of Rabbit Reticulocyte Lysate only, it would be interesting to repeat this experiment using an RNA sample from a tissue virtually devoid of DCA-*N*-MT activity (e.g. *Arabidopsis* leaves) to ensure that a significant difference with soybean RNA samples would indeed be detectable. The utilisation of a radiolabelled amino acid (usually ^{35}S-methionine) during the *in vitro* translation would also improve the determination of DCA-*N*-MT specific activity, as it would give an approximation of the quantity of protein synthesised during the process, hence a more precise calculation of the specific activity.

It was then decided to pursue the induction of DCA-*N*-MT activity as a possible means of obtaining protein sequence and ultimately cloning the corresponding cDNA. DCA induction of DCA-*N*-MT activity was applied to the protein purification scheme developed in Chapter 4 and the comparison of DCA-treated and control samples by 1D-PAGE, together with kinetic data, suggested that the increased enzyme activity was due to *de novo* protein synthesis of DCA-*N*-MT (figure 5.7). The 53 kDa band tentatively identified in Chapter 4 as DCA-*N*-MT was increased in intensity in proteins purified from DCA-treated soybean roots. Following DCA treatment, no additional proteins were detected by 1D PAGE, but given the limited resolution of this technique, a more detailed quantitative and qualitative analysis was performed using 2D-DiGE.

Several soybean proteins were identified as being induced by DCA treatment. These include three enzymes which are associated with oxidative stress responses: ascorbate peroxidase, superoxide dismutase and GST, suggesting that DCA could have imposed a mild oxidative stress on the soybean plants. This is not surprising, since herbicide-induced oxidative stress has been reported previously (e.g. Cummins *et al.*, 1999). The only induced protein identified of ca. 52 kDa was UDP-glucose pyrophosphorylase (see below). Unless this represents a "moonlighting protein" (Jeffery, 1999; Copley, 2003), no obvious candidate for DCA-*N*-MT was identified. It should be noted however, that several protein spots were not of sufficient abundance or similarity to proteins in sequence databases to be identifiable in the first experiments. Further 1D and 2D analysis of differentially-regulated proteins and of proteins in the 53 kDa size range from active, partially-purified fractions is currently being undertaken and it is hoped that DCA-*N*-MT might yet be identified by these means.

Interestingly, phosphoglucomutase was somewhat down-regulated by DCA treatment. Combined with the DCA induction of UDP-glucose pyrophosphorylase, this could lead to the increased formation of UDP glucose (UDPG) in soybean roots, as outlined in figure 5.9. In future studies, this could be tested by enzyme-linked spectrophotometric measurement of UDPG, glucose-1-phosphate and glucose-6-phosphate in perchloric acid extracts of treated and untreated tissue (Schluepmann *et al.*, 2003). A potential increase in UPDG availability is notable since UDPG is the co-substrate for DCA-*N*-GT and DCA treatment could therefore increase detoxification of DCA by the minor glucosylation route in soybean identified in

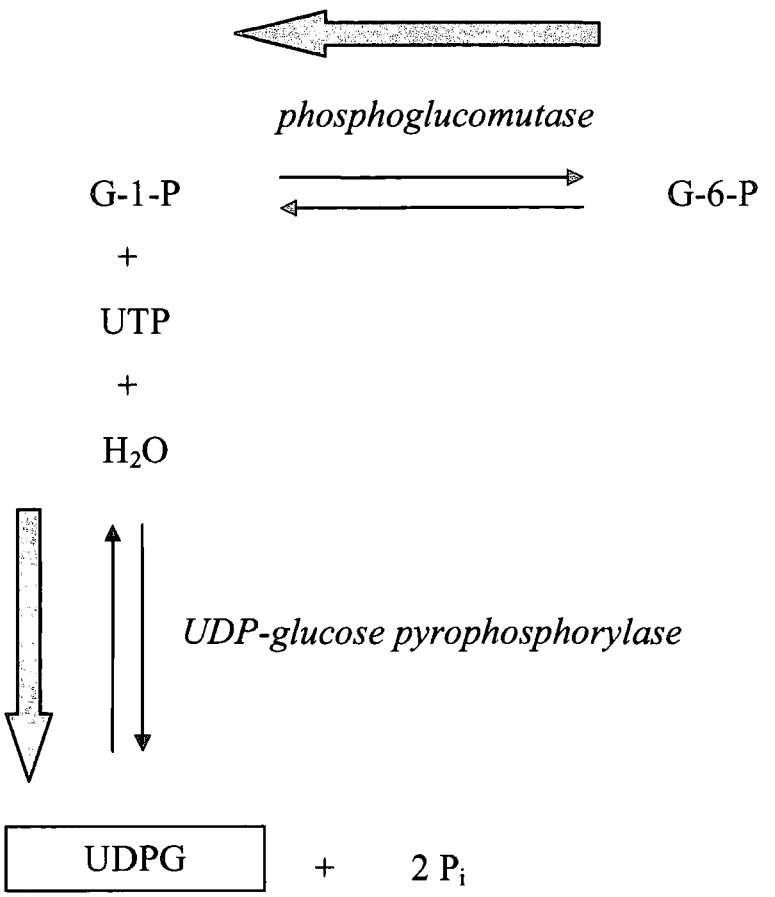


Figure 5.9: Hypothetical scheme showing the effect of DCA pre-treatment on enzymes involved in the synthesis of UDP-glucose.

Phosphoglucomutase catalyses the reversible conversion of glucose-1-phosphate (G-1-P) into glucose-6-phosphate (G-6-P) and uridine diphosphate glucose (UDP-glucose or UDPG) is synthesised from G-1-P and UTP in a reversible reaction catalysed by UDP-glucose pyrophosphorylase. DiGE analysis of soybean roots treated with 100 μM DCA indicated that the abundance of UDP glucose pyrophosphorylase is increased in response to treatment and the abundance of phosphoglucomutase is decreased. If the changes in protein abundance are reflected in enzyme activity, flux would be directed towards the formation of UDPG, as shown by the block arrows.

Chapter 3. Unfortunately, owing to time constraints, the induction of DCA-*N*-GT activity was not studied in this project, but this would be an important route to pursue in future. It also seems plausible that UDP-glucose pyrophosphorylase co-purifies with DCA-*N*-MT in both purification schemes developed and although gel filtration demonstrated that the native molecular weight of DCA-*N*-MT is 52 ± 2 kDa, UDP glucose pyrophosphorylase probably accounts for a significant proportion of the 52 kDa band observed in 1D-PAGE.

The potential for DCA to increase carbon flux in favour of UDPG formation raises the question of whether the synthesis of M-CoA, the co-substrate of DCA-*N*-MT, is also induced by DCA pre-treatment. In dicotyledonous plants, M-CoA is synthesised by a malonyl-CoA synthetase in mitochondria (Gueguen *et al.*, 2000), and by prokaryotic and eukaryotic forms of acetyl-coenzyme A carboxylase (ACCase) in plastids and cytosol, respectively (reviewed by Sasaki *et al.*, 1995). Cytosolic M-CoA is most likely to contribute to co-substrate production for DCA-*N*-MT which appears to be a soluble cytosolic enzyme. Unfortunately, as a protein of > 200 kDa, cytosolic ACCase is too large to be resolved readily in 2D gels and it would by unlikely to be detected by DiGE. Although no simple method is available for the measurement of malonyl-CoA in plant extracts, ACCase can be measured relatively easily in desalted ammonium sulphate extracts using a radiometric assay (Shukla *et al.*, 1997) and this would be an interesting topic for further study.

5.7. Summary

Pre-treatment of soybean roots with the xenobiotic DCA prior to enzyme extraction increased DCA-*N*-MT activity, a finding that has not been reported previously. Kinetic comparisons of DCA-*N*-MT in crude extracts from DCA-treated and control tissues and the parallel purification of proteins from the same material strongly suggested that this augmentation in activity was due to *de novo* protein synthesis. DCA induction of activity was maintained throughout the purification procedure and analysis of partially-purified proteins from DCA-treated and control tissues using 1D gel electrophoresis suggested that a protein of approximately 53 kDa was a candidate for DCA-*N*-MT. 2D DiGE analysis revealed that a number of proteins increased in abundance in response to DCA pre-treatment but these did not include a protein which could be confidently identified as DCA-*N*-MT. At present, it

cannot be excluded that the DCA-*N*-MT protein is not induced and that the increase in activity observed following DCA pre-treatment of roots is due to other factors. These are nevertheless interesting and novel findings, providing the basis for a wealth of future studies which will shed light on the effects of DCA treatment on plant metabolism and through further proteomic analysis could ultimately lead to molecular characterisation of this detoxification enzyme.

Chapter 6

Discussion

The overall aim of this project was to study soybean DCA-*N*-MT in order to obtain information which would lead to its molecular identification and to understand better the role of this enzyme in the detoxification of the reference xenobiotic DCA. Together with GSTs and GTs, MTs are involved in Phase II of xenobiotic conjugation in plants but remarkably little is known about the relative importance and identity of malonyltransferases.

6.1. The metabolic fate of DCA in soybean

The first task undertaken was to study the fate of DCA in intact soybean plants in order to make comparisons with DCA metabolism in other plant systems. In chapter 3, it was shown that DCA was mainly metabolised within the roots with very little translocation to aerial parts of plants (section 3.2.1). Consistent with the observation that DCA-*N*-MT activity was highest in the roots, the principal DCA metabolite formed was an *N*-malonyl-conjugate, although a very small amount of *N*-glucosyl DCA was also detected (figure 3.6). Interestingly, M-DCA was exported out of the cells, into the external growth medium (figure 3.5). This was largely consistent with previous studies using soybean cell cultures (Scheel *et al.*, 1984; Schmitt *et al.*, 1985; Winkler and Sandermann, 1989; Sandermann *et al.*, 1991; Schmidt *et al.*, 1994) and excised leaves (Gareis *et al.*, 1992) although the relative amounts of the different conjugates varied between cultivars: in the only other study where intact soybean plants were utilised, formation of a greater proportion of G-

DCA was correlated with increased translocation to the shoot and increased bound residue formation (Bockers *et al.*, 1994). In a parallel study, DCA was found to be metabolised to both the *N*-glucosyl and *N*-malonyl conjugates in *Arabidopsis*, although the glucosyl conjugate predominated (Lao *et al.*, 2003; Appendix 1). Interestingly, both M-DCA and G-DCA were exported from *Arabidopsis* root cultures. Similarly, DCA has been shown previously to be metabolised by both glucosylation and malonylation in carrot cell cultures (Schmidt *et al.*, 1994), with export of the malonyl conjugate. In contrast however, DCA was metabolised predominantly to G-DCA in wheat, with a larger proportion of bound residues being formed (Bockers *et al.*, 1994).

There are several possible explanations for the system-, species-, and cultivar-specific differences in DCA metabolism. In the present study (section 3.2.5) and that of Schmidt *et al.* (1995), the metabolic fate of DCA correlated well with the endogenous complement of transferase enzymes. However, the predominant route for detoxification could also reflect the availability of the co-substrates M-CoA and UDPG: soybean might be expected to produce more M-CoA for lipid and flavonoid synthesis compared to *Arabidopsis* and wheat, which might favour a detoxification route involving glucosylation (see below). In agreement with this, peanut, a plant which produces large amounts of lipid, is a rich source of multiple malonyltransferases (Matern, 1984). Moreover, it might be expected that the structural complexity of intact plants offers more potential DCA sinks than suspension cultures, which do not possess a vascular system and extensive cell wall material which would facilitate the formation of bound residues.

6.2. Export of M-DCA and its implications for the environment

The export of DCA conjugates in soybean, *Arabidopsis* and carrot is in marked contrast to the "classical" model of xenobiotic detoxification, in which xenobiotic conjugates are sequestered in the vacuole. However, it cannot be excluded that a small proportion of the M-DCA was stored in the vacuole in both species. At any time-point after being treated with radiolabelled DCA, the amount of M-DCA within the roots did not vary greatly (table 3.1). This observation suggests the possible existence of mechanisms capable of regulating the intracellular level of M-DCA. Since the malonyl-conjugate was only detected in the growth medium after

30 min and considering the high specific activity of DCA-*N*-MT in soybean roots, it is tempting to hypothesise that M-DCA accumulated rapidly within the vacuole up to a maximal level above which compartmentation was directed towards export across the plasma membrane. In many cases, the vacuole is only a temporary storage site for conjugated natural products and xenobiotics. For example, glutathione conjugates are rapidly metabolised following transport into the vacuole (Schröder, 1997) and therefore do not accumulate over time. In contrast, plants apparently do not possess mechanisms for the degradation of M-DCA (Matern *et al.*, 1984), suggesting that the vacuole cannot act as an infinite sink for this conjugate. In this context, it would be interesting to isolate whole vacuoles from soybean roots treated with radiolabelled DCA to determine what proportion (if any) of the malonyl-conjugate was stored in the vacuole and furthermore to use isolated vacuoles to investigate uptake of radiolabelled M-DCA and determine whether a transport system capable of sequestering M-DCA was present.

According to its physico-chemical properties, the passive transport of M-DCA through biological membranes is very unlikely. In agreement with this, preliminary experiments involving the utilisation of transport inhibitors suggested the presence of an active transporter, the identity of which is currently unknown (section 3.2.6.2). It has been shown that ABC transporters and secondary transport systems mediate the compartmentation of both xenobiotic metabolites and endogenous substances (Klein *et al.*, 1996; Klein *et al.*, 2000; Bartholomew *et al.*, 2002), and both these classes of transporters are candidates for the plasma membrane and vacuolar transport of DCA conjugates. Experimental approaches to characterising M-DCA transport are discussed in more detail in Chapter 3.

In the present study, DCA-treated soybean roots were shown to transport M-DCA out of the roots to the external growth medium. Once exported, cross-feeding studies demonstrated that M-DCA was not readily taken up and moreover did not appear to be metabolised further by extracellular enzymes. This raises the question of what happens in soil-grown plants. Unlike the semi-sterile growth medium used in hydroponics, soil contains a wide range of micro-organisms capable of metabolising complex organic molecules. This has important implications for the fate of M-DCA in soil. From a bioremediation viewpoint, it would be interesting to assess the toxicity and the persistence of the conjugate, relative to its parent xenobiotic DCA. One of the main reasons why DCA is a pollutant is that it binds

covalently to soil particles (Thorn *et al.*, 1996) and is very difficult to degrade for micro-organisms (El-Fantroussi, 2000). Since soybean plants can readily detoxify DCA and excrete its malonylated conjugate (Lao *et al.*, 2003), it would be interesting to determine whether M-DCA could be biodegraded in soil. The white-rot fungus *Phanerochaete chrysosporium* being capable of mineralising DCA (May *et al.*, 1997), an obvious approach would be to test the capacity of this micro-organism to degrade M-DCA. If this was possible, the phytoremediation of DCA-contaminated soils with soybean plants associated with *P. chrysosporium* would be plausible.

The export of a malonylated compound is interesting in the context of rhizosphere signalling by legumes (reviewed by Jain and Nainawatee, 2002). Firstly, the *nod* factors involved in nitrogen fixation have been well characterised and include an *O*-malonylated flavonoid (Pueppke *et al.*, 1998) which must be exported across the plasma membrane, either via a membrane-bound transporter or by secretion. The existence of a root plasma membrane transport system for endogenous malonylated compounds is interesting with respect to the export of M-DCA. Secondly, at concentrations as low as nanomolar to micromolar levels, flavonoids exuded by plants influence chemotaxis, regulate soil microbes and promote spore germination in mycorrhizal fungi. It is very tempting to propose that M-DCA is exported by a mechanism similar to that used by conjugated flavonoids.

6.3. Towards the isolation of DCA-*N*-MT

Having established that malonylation plays an important role in the detoxification of DCA in several plant species, classical approaches were undertaken to purify and characterise DCA-*N*-MT in Chapter 4. Two purification schemes were tested which resulted in the enrichment of DCA-*N*-MT activity, but neither was sufficient to achieve purification to homogeneity. Had more time been available, the development of an affinity chromatography step, for example, using a DCA-coupled matrix would have been attempted. This has the potential to afford higher purity, by removing a large proportion of proteins that have different binding properties to DCA-*N*-MT. This purification step would then have been incorporated in the purification protocol described in section 4.2.2.3. (procedure 2.2).

In common with other phase II detoxification enzymes, the activity of DCA-*N*-MT was increased by pre-treatment with its substrate (section 5.2). Kinetic studies suggested that this increase was possibly due to *de novo* protein synthesis. This was an important finding because it provided a potential route to the identification of DCA-*N*-MT. Firstly, regardless of the mechanism underlying increased DCA-*N*-MT activity, this phenomenon could be used to assist purification, as it would be easier to monitor activity during the different chromatographic steps. Secondly, the apparent induction of DCA-*N*-MT could be used to identify the protein. Following gel filtration, the native molecular weight of DCA-*N*-MT was found to be 52 ± 2 kDa (section 4.2.2.3.2). In agreement with this, a strong band of this size was observed in 1D denaturing gels of the partially-purified enzyme. The intensity of this band increased following DCA pre-treatment of the roots, suggesting that it did indeed represent DCA-*N*-MT. Since 1D SDS-PAGE is limited in its ability to separate protein mixtures, a 2D approach to identify differentially-regulated proteins was adopted. 2D-DiGE analysis of partially-purified DCA-*N*-MT revealed several proteins whose abundance was altered by pre-treatment with DCA (table 5.2). Disappointingly, no obvious candidate for DCA-*N*-MT was identified, although several protein spots were not of sufficient abundance to be identified or did not have sufficient homology to proteins in the public databases, possibly because a full genome sequence for soybean is not yet available. One protein in the correct molecular weight range was differentially regulated, but this was identified as UDP-glucose pyrophosphorylase (51.8 kDa). Since the amount of this protein increased ca. three-fold in response to DCA pre-treatment, this could have accounted for the increased intensity of the 52 kDa band observed in 1D gels and it is therefore unclear whether the amount of DCA-*N*-MT protein also increases. If the increase in DCA-*N*-MT activity is due to factors other than an increase in the amount of the enzyme itself, it would not be identified by DiGE. An important future experiment therefore, is to separate partially-purified DCA-*N*-MT in a preparative gel, cut the band corresponding to ca. 52 kDa proteins out, separate these proteins in a conventional 2D gel and analyse all the proteins of ca. 52 kDa.

6.4. Importance of the co-substrates

The induction of UDP-glucose pyrophosphorylase by DCA is interesting for two reasons: firstly, there is the possibility that this enzyme has two different catalytic activities: the phenomenon of "moonlighting" proteins is well described and such proteins include enzymes of sugar metabolism (Jeffery, 1999). For example, phosphoglucose isomerase, a major enzyme involved in glycolysis that catalyses the interconversion of glucose 6-phosphate and fructose 6-phosphate, moonlights as neuroleukin, autocrine motility factor and differentiation and maturation mediator (Jeffery, 1999; Copley, 2003). Since UDP-glucose pyrophosphorylase is available commercially, it would be straightforward to test whether it possesses DCA-*N*-MT activity. However, the existence of different *N*-MT isoforms with differing substrate specificity towards endogenous compounds suggests that DCA-*N*-MT belongs to a distinct gene family of *N*-malonyltransferases, rather than arising from catalytic flexibility of an enzyme that has a central role in carbon metabolism. Secondly, as discussed in Chapter 5, the simultaneous up-regulation of UDP-glucose pyrophosphorylase, and down-regulation of phosphoglucomutase could result in increased synthesis of UDPG, thus favouring the formation of G-DCA. This begs the question of whether M-CoA synthesis is also increased by DCA pre-treatment.

In the cytosol, where M-CoA is needed for fatty acid elongation and for the biosynthesis of phytoalexins and flavonoids (Sasaki *et al.*, 1995; Reverdatto *et al.*, 1999), M-CoA production is catalysed by the eukaryotic form of acetyl-CoA carboxylase (ACCase; Konishi and Sasaki, 1994). In addition to being indispensable for the biosynthesis of fatty acids, M-CoA is the precursor of tetrahydroxychalcone (THC), a central compound involved in the formation of flavonoids, including anthocyanins, flavones, flavonols and isoflavonoids. THC is synthesised when three molecules of M-CoA are condensed with 4-coumaroyl-CoA via the action of chalcone synthase. The treatment of alfalfa cell suspension cultures with fungal and yeast elicitors induced ACCase activity several-fold at the transcriptional level, suggesting that constitutive levels of M-CoA are insufficient when the synthesis of both fatty acids and additional secondary metabolites such as phyoalexins is necessary (Shorrosh *et al.*, 1994). It is thus conceivable that DCA-treatment of soybean roots would have induced the ACCase activity to compensate for the higher

M-CoA requirement for M-DCA synthesis. Attempts to assess M-CoA concentration would have been useful to find out whether M-CoA was present in sufficient quantities in control and DCA-treated soybean roots and if M-CoA limitation could have affected the fate of DCA.

6.5. Xenobiotic detoxification and secondary metabolism, where does the boundary lie?

The examples cited above highlight parallels in the pathways involved in secondary metabolism and in the detoxification of xenobiotics. Both pathways involve formation of conjugates and their transport into different subcellular compartments. This is not surprising since many secondary products can be toxic to the plant that produces them and thus require sequestration in the vacuole, a feature that is related to xenobiotic detoxification. These similarities raise a question that, to date, has not been fully answered, namely: are these pathways independent, identical or partially shared? Some important comparisons (similarities and differences) between the formation and transport of natural and xenobiotic malonosides can be made based on the work reported here and that in the literature.

Since DCA is not naturally present in the environment, and herbicides have only been used systematically for the last 50 years, the mechanisms involved in its degradation must have other functions related to the metabolism and transport of endogenous compounds. In fact, several *N*-MTs have been reported to accept more than one substrate. For example, ACC-*N*-MT and D-amino acid *N*-MT seem to be correlated in mung bean (Liu *et al.*, 1983; Guo *et al.*, 1993) and in tomato leaves, a tryptophan-*N*-MT could accept both D- and L-tryptophan (Wu *et al.*, 1995). In soybean cell cultures, Sandermann and co-workers (1991) studied the substrate specificity of DCA-*N*-MT using various xenobiotics and secondary metabolites and showed that the enzyme had a preference for chlorinated anilines but could also accept 7-amino-4-methyl coumarin as a substrate, with a conversion rate of 70 % of that of DCA. However, when a range of other natural compounds, including certain D- and L-amino acids and ACC were tested, DCA-*N*-MT activity was less than 3 % relative to the activity for DCA. This flexibility in substrate acceptance is reminiscent of the properties of GSTs and GTs: both families of enzymes possess two domains, one that is highly specific for glutathione and UDP-glucose

respectively and a second domain that can accept a wide range of substrates, including herbicides. This provides a structural explanation for the presence of xenobiotic-metabolising activities in plants. Moreover, as discussed above, transporters involved in the compartmentation of endogenous compounds can also transport xenobiotics, suggesting that identical transporters are possibly responsible for both xenobiotic and endogenous compound metabolisms.

Had the enzyme been purified with higher yields or, had it been possible to obtain a recombinant protein, the sensitive and reproducible DCA-*N*-MT assay developed during this project would have allowed a reliable screening of DCA-*N*-MT activity with a range of endogenous compounds. The results would be highly informative and could point towards a possible secondary metabolism pathway shared by xenobiotics and endogenous molecules.

6.6. Future work

The aim of this thesis was to study soybean DCA-*N*-MT in order to understand better its role in the detoxification of the reference xenobiotic, DCA. *In planta* metabolism studies demonstrated that after DCA treatment, the major degradation metabolite synthesised in soybean was a malonyl-conjugate, M-DCA. In contrast to most xenobiotic detoxification routes which direct the compartmentation of xenobiotic metabolites to the vacuole, M-DCA was transported out of the roots. Preliminary studies presented in this thesis suggested the involvement of a secondary transporter but more work will be necessary to identify the protein responsible for this excretion. Since this has never been studied, one of the important points to investigate in the future would be to identify a plasma membrane transporter that allows the export, not only of M-DCA but maybe of other xenobiotic metabolites. The isolation of this transporter would lead to the unravelling of the mechanisms underlying this export.

Since malonylation was demonstrated to be the main DCA detoxification route in soybean, the purification of DCA-*N*-MT was attempted in order to obtain sequence information. A reliable and sensitive assay for the detection of DCA-*N*-MT was developed and was employed to purify the enzyme up to 400-fold. However, no sequence information was obtained. Had the gene encoding DCA-*N*-MT been identified and cloned, the expression of the corresponding recombinant

protein would have opened the way to a wide range of studies and its sequence could have been used to identify the gene family to which DCA-*N*-MT belongs. For example, basic kinetics studies could have been performed, including the determination of the enzyme's substrate specificities for other xenobiotics and endogenous compounds such as ACC and D-amino acids. The results would be invaluable to determine whether DCA-*N*-MT is capable of catalysing the detoxification of both endogenous and xenobiotic compounds. For the first time, DCA-treatments of soybean roots was shown to increase DCA-*N*-MT activity. The availability of DCA-*N*-MT sequence would be useful to determine the level (transcriptional or the translational) at which DCA-*N*-MT activity was induced and would have been a means to find out whether this induction was due to *de novo* synthesis of the enzyme itself or due to other factors, an information of importance for the understanding of induction mechanisms following xenobiotic treatments. Since M-DCA was shown here to be exported from soybean roots, as opposed to the general belief that xenobiotic conjugates are compartmentalised within the vacuole, the availability of recombinant DCA-*N*-MT would permit the synthesis of high quantities of M-DCA, which would be required for transport studies (see section 3.3). The results obtained would contribute towards the unravelling of the mechanisms involved in the export of xenobiotic conjugates, a detoxification route that has not been studied to date.

The answers to these questions will only be resolved by the cloning and characterisation of DCA-*N*-MT and the eventual identification of M-DCA transporters. This study has been an important step forward in achieving this.

REFERENCES

Alfenito MR, Souer E, Goodman CD, Buell R, Mol J, Koes R and Walbot V (1998) Functional complementation of anthocyanin sequestration in the vacuole by widely divergent glutathione *S*-transferases. Plant Cell **10:** 1135-1150

Alla MMN (1995) Glutathione Regulation of Glutathione-*S*-Transferase and Peroxidase-Activity in Herbicide-Treated *Zea mays*. Plant Physiology and Biochemistry **33:** 185-192

Alla MMN and Hassan NM (1998) Efficacy of exogenous GA(3) and herbicide safeners in protection of *Zea mays* from metolachlor toxicity. Plant Physiology and Biochemistry **36:** 809-815

Aoki T, Akashi T and Ayabe S (2000) Flavonoids of leguminous plants: Structure, biological activity, and biosynthesis. Journal of Plant Research **113:** 475-488

Barrett M, Polge N, Baerg R, Bradshaw L and Poneleit C (1997) Role of cytochrome P-450 in herbicide metabolism and selectivity and multiple herbicide metabolizing cytochrome P-450 activities in maize. *In* KK Hatzios, ed, Regulation of enzymatic systems detoxifying xenobiotics in plants. Kluwer Academic Publishers, The Netherlands, pp 35-50

Bartholomew DM, Van Dyk DE, Lau SMC, O'Keefe DP, Rea PA and Viitanen PV (2002) Alternate energy-dependent pathways for the vacuolar uptake of glucose and glutathione conjugates. Plant Physiology **130:** 1562-1572

Barz W and Mackenbrock U (1994) Constitutive and elicitation induced metabolism of isoflavones and pterocarpans in chickpea (*Cicer arietinum*) cell suspension cultures. Plant Cell Tissue and Organ Culture **38:** 199-211

Bénichou M, Martinezreina G, Romojaro F, Pech JC and Latche A (1995) Partial-purification and properties of a 36-kda 1-aminocyclopropane- 1-carboxylate *N*-malonyltransferase from mung bean. Physiologia Plantarum **94:** 629-634

Blanchard JS (1984) Buffers for enzymes. *In* WB Jakoby, ed, Enzyme purification and related techniques. Part C., Vol 104. Academic Press, pp 404-414

Blatt MR, Beilby MJ and Tester M (1990) Voltage Dependence of the Chara Proton Pump Revealed by Current-Voltage Measurement During Rapid Metabolic Blockade with Cyanide. Journal of Membrane Biology **114:** 205-223

Bockers M, Rivero C, Thiede B, Jankowski T and Schmidt B (1994) Uptake, translocation, and metabolism of 3,4-dichloroaniline in soybean and wheat plants. Zeitschrift Fur Naturforschung C-a Journal of Biosciences **49:** 719-726

Bolwell GP, Bozak K and Zimmerlin A (1994) Plant Cytochrome-P450. Phytochemistry **37:** 1491-1506

Bowles D (2002) A multigene family of glycosyltransferases in a model plant, *Arabidopsis thaliana*. Biochemical Society Transactions **30:** 301-306

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Analytical Biochemistry **72:** 248-254

Breton C and Imberty A (1999) Structure/function studies of glycosyltransferases. Current Opinion in Structural Biology **9:** 563-571

Breton C, Mucha J and Jeanneau C (2001) Structural and functional features of glycosyltransferases. Biochimie **83:** 713-718

Briggs GG, Bromilow RH and Evans AA (1982) Relationships between Lipophilicity and Root Uptake and Translocation of Non-Ionized Chemicals by Barley. Pesticide Science **13:** 495-504

Bromilow RH and Chamberlain K (1995) Principles governing uptake and transport of chemicals. *In* S Trapp, JD McFarlane, eds, Plant contamination: modelling and simulation of organic chemical processes. CRC Press, London, pp 37-68

Brunow G, Raiskila S and Sipila J (1998) The incorporation of 3,4-dichloroaniline, a pesticide metabolite, into dehydrogenation polymers of coniferyl alcohol (DHPs). Acta Chemica Scandinavica **52:** 1338-1342

Campbell JA, Davies GJ, Bulone V and Henrissat B (1997) A classification of nucleotide-diphospho-sugar glycosyltransferases based on amino acid sequence similarities. Biochemical Journal **326:** 929-942

Chamberlain K, Evans AA and Bromilow RH (1996) 1-octanol/water partition coefficient (K-ow) and pK(a) for ionisable pesticides measured by a pH-metric method. Pesticide Science **47:** 265-271

Charnock SJ, Henrissat B and Davies GJ (2001) Three-dimensional structures of UDP-sugar glycosyltransferases illuminate the biosynthesis of plant polysaccharides. Plant Physiology **125:** 527-531

Chick WSH and Leung PC (1997) Immunopurification and characterization of a 40-kD 1- aminocyclopropane-1-carboxylic acid N-malonyltransferase from mung bean seedling hypocotyls. Plant Physiology **113:** 119-126

Cole DJ, Cummins I, Hatton PJ, Dixon D and Edwards R (1997) Glutathione transferases in crops and major weeds. *In* KK Hatzios, ed, Regulation of enzymatic systems detoxifying xenobiotics in plants. Kluwer Academic Publishers, The Netherlands, pp 139-154

Cole DJ and Edwards R (2000) Secondary metabolism of agrochemicals in plants. *In* T Roberts, ed, Metabolism of agrochemicals in plants. John Wiley & Sons Ltd, London, pp 107-154

Coleman JOD, BlakeKalff MMA and Davies TGE (1997) Detoxification of xenobiotics by plants: Chemical modification and vacuolar compartmentation. Trends in Plant Science **2:** 144-151

Copley SD (2003) Enzymes with extra talents: moonlighting functions and catalytic promiscuity. Curr Opin Chem Biol **7:** 265-272

Cornish-Bowden A and Eisenthal R (1974) Statistical considerations in the estimation of enzyme kinetic parameters by the direct linear plot and other methods. Biochemical Journal **139:** 721-730

Cummins I, Cole DJ and Edwards R (1999) A role for glutathione transferases functioning as glutathione peroxidases in resistance to multiple herbicides in black-grass. Plant Journal **18:** 285-292

Dixon DP, Lapthorn A and Edwards R (2002) Plant glutathione transferases. Genome Biology **3:** 3004.3001-3004.3010

Ebing W, Haque A, Schuphan I, Harms H, Langebartels C, Scheel D, Vondertrenck KT and Sandermann H (1984) Ecochemical Assessment of Environmental Chemicals - Draft Guideline of the Test Procedure to Evaluate Metabolism and Degradation of Chemicals by Plant-Cell Cultures. Chemosphere **13:** 947-957

Edwards R, Dixon DP and Walbot V (2000) Plant glutathione *S*-transferases: enzymes with multiple functions in sickness and in health. Trends in Plant Science **5:** 193-198

El-Fantroussi S (2000) Enrichment and molecular characterization of a bacterial culture that degrades methoxy-methyl urea herbicides and their aniline derivatives. Applied and Environmental Microbiology **66:** 5110-5115

Fernandez-Canon JM and Penalva MA (1998) Characterization of a fungal maleylacetoacetate isomerase gene and identification of its human homologue. Journal of Biological Chemistry **273:** 329-337

Fraissinet-Tachet L, Baltz R, Chong J, Kauffmann S, Fritig B and Saindrenan P (1998) Two tobacco genes induced by infection, elicitor and salicylic acid encode glucosyltransferases acting on phenylpropanoids and benzoic acid derivatives, including salicylic acid. FEBS Letters **437:** 319-323

Frear DS and Swanson HR (1970) The biosynthesis of *S*-(4-ethylamino-6-isopropylamino-*S*-5-triazino) glutathione: partial purification and properties of a glutathione *S*-transferase from corn. Phytochemistry **9:** 2123-2132

Freitag D, Scheunert I, Klein W and Korte F (1984) Long-term fate of 4-chloroaniline in soil and plants under outdoor conditions. A contribution to terrestrial ecotoxicology of chemicals. Journal of Agricultural and Food Chemistry **32:** 203-207

Fujiwara H, Tanaka Y, Yonekura-Sakakibara K, Fukuchi-Mizutani M, Nakao M, Fukui Y, Yamaguchi M, Ashikari T and Kusumi T (1998) cDNA cloning, gene expression and subcellular localization of anthocyanin 5-aromatic acyltransferase from *Gentiana triflora*. Plant Journal **16:** 421-431

Gaedeke N, Klein M, Kolukisaoglu U, Forestier C, Muller A, Ansorge M, Becker D, Mamnun Y, Kuchler K, Schulz B, Mueller-Roeber B and Martinoia E (2001) The *Arabidopsis thaliana* ABC transporter AtMRP5 controls root development and stomata movement. EMBO journal **20:** 1875-1887

Gaillard C, Dufaud A, Tommasini R, Kreuz K, Amrhein N and Martinoia E (1994) A Herbicide Antidote (Safener) Induces the Activity of Both the Herbicide Detoxifying Enzyme and of a Vacuolar Transporter for the Detoxified Herbicide. FEBS Letters **352:** 219-221

Gareis C, Rivero C, Schuphan I and Schmidt B (1992) Plant-Metabolism of Xenobiotics - Comparison of the Metabolism of 3,4-Dichloroaniline in Soybean Excised Leaves and Soybean Cell- Suspension Cultures. Zeitschrift Fur Naturforschung C-a Journal of Biosciences **47:** 823-829

Gueguen V, Macherel D, Jaquinod M, Douce R and Bourguignon J (2000) Fatty acid and lipoic acid biosynthesis in higher plant mitochondria. Journal of Biological Chemistry **275:** 5016-5025

Guengerich FP (2003) Cytochrome P450 oxidations in the generation of reactive electrophiles: epoxidation and related reactions. Archives of Biochemistry and Biophysics **409:** 59-71

Guo L, Arteca RN, Phillips AT and Liu Y (1992) Purification and characterization of 1-aminocyclopropane-1-carboxylate *N*-malonyltransferase from etiolated mung bean hypocotyls. Plant Physiology **100:** 2041-2045

Guo L, Phillips AT and Arteca RN (1993) Amino acid *N*-malonyltransferase from mung beans: Action on 1-aminocyclopropane-1-carboxylic acid and D-phenylalanine. Journal of Biological Chemistry **268:** 25389-25394

Guo L, Phillips AT and Arteca RN (1993) Relationship between ACC *N*-malonyltransferase and D-amino acid *N*-malonyltransferase in etiolated mung bean hypocotyls. Joint Annual Meeting of the American Society of Plant Physiologists and the Canadian Society of Plant Physiologists **102:** 55

Hall JC, Wickenden JS and Yau KYF (2001) Biochemical conjugation of pesticides in plants and microorganisms: an overview of similarities and divergences. *In* Pesticide Transformations in Plants and Microorganisms: Similarities and Divergences. American Chemical Society, pp 89

Harborne JB (1986) The Natural Distribution in Angiosperms of Anthocyanins Acylated with Aliphatic Dicarboxylic-Acids. Phytochemistry **25:** 1887-1894

Harms H and Langebartels C (1986) Standardized Plant-Cell Suspension Test Systems for an Ecotoxicologic Evaluation of the Metabolic-Fate of Xenobiotics. Plant Science **45:** 157-165

Heim K, Schuphan I and Schmidt B (1994) Behavior of C-14 4-Nitrophenol and C-14 3,4-Dichloroaniline in Lab Sediment-Water Systems .1. Metabolic-Fate and Partitioning of Radioactivity. Environmental Toxicology and Chemistry **13:** 879-888

Heim K, Schuphan I and Schmidt B (1995) Behavior of C-14 4-Nitrophenol and C-14 3,4-Dichloroaniline in Laboratory Sediment-Water Systems .2. Desorption Experiments and Identification of Sorptive Fraction. Environmental Toxicology and Chemistry **14:** 755-761

Henderson PJF (1992) Statistical analysis of enzyme kinetic data. *In* R Eisenthal, MJ Danson, eds, Enzyme assays. A practical approach., ed, IRL Press. Oxford University Press, Oxford, pp 277-316

Higgins CF (1992) ABC-transporters: from microorganisms to man. Annual Review of Cell Biology **8:** 67-113

Hinder B, Schellenberg M, Rodon S, Ginsburg S, Vogt E, Martinoia E, Matile P and Hortensteiner S (1996) How plants dispose of chlorophyll catabolites - Directly energized uptake of tetrapyrrolic breakdown products into isolated vacuoles. Journal of Biological Chemistry **271:** 27233-27236

Hoagland RE and Zablotowicz RM (2001) The Role of Plant and Microbial Hydrolytic Enzymes in Pesticide Metabolism. *In* JC Hall, RE Hoagland and RM Zablotowicz, eds, Pesticide Transformations in Plants and Microorganisms: Similarities and Divergences. ACS Symp. Ser. 777, American Chemical Society, pp 58-88

Hoffmann L, Maury S, Martz F, Geoffroy P and Legrand M (2003) Purification, cloning, and properties of an acyltransferase controlling shikimate and quinate ester intermediates in phenylpropanoid metabolism. Journal of Biological Chemistry **278:** 95-103

Höhl HU and Barz W (1995) Metabolism of the insecticide Phoxim in plants and cell suspension cultures of Soybean. Journal of Agricultural and Food Chemistry **43:** 1052-1056

Hörtensteiner S (1998) NCC malonyltransferase catalyses the final step of chlorophyll breakdown in rape (*Brassica napus*). Phytochemistry **49:** 953-956

Hu YN and Walker S (2002) Remarkable structural similarities between diverse glycosyltransferases. Chemistry & Biology **9:** 1287-1296

Hunaiti AA and Ali BR (1991) The Induction of Chickpea Glutathione-S-Transferase by Oxadiazon. Phytochemistry **30:** 2131-2134

Incledon BJ and Hall JC (1997) Enzymatic de-esterification of xenobiotics in plants. *In* KK Hatzios, ed, Regulation of enzymatic systems detoxifying xenobiotics. Kluwer Academic Publishers, Netherlands, pp 67-82

Jain V and Nainawatee HS (2002) Plant flavonoids: Signals to legume nodulation and soil microorganisms. Journal of Plant Biochemistry and Biotechnology **11:** 1-10

Jakobsson PJ, Morgenstern R, Mancini J, Ford-Hutchinson A and Persson B (1999) Common structural features of MAPEG - a widespread superfamily of membrane associated proteins with divergent functions in eicosanoid and glutathione metabolism. Protein Science **8:** 689-692

Jasinski M, Stukkens Y, Degand H, Purnelle B, Marchand-Brynaert J and Boutry M (2001) A plant plasma membrane ATP binding cassette-type transporter is involved in antifungal terpenoid secretion. Plant Cell **13:** 1095-1107

Jeffery CJ (1999) Moonlighting proteins. Trends Biochem Sci **24:** 8-11

Jones P and Vogt T (2001) Glycosyltransferases in secondary plant metabolism: tranquilizers and stimulant controllers. Planta **213:** 164-174

Kapitonov D and Yu RK (1999) Conserved domains of glycosyltransferases. Glycobiology **9:** 961-978

Keegstra K and Raikhel N (2001) Plant glycosyltransferases. Current Opinion in Plant Biology **4:** 219-224

King RW, Lustig KD, Stukenberg PT, McGarry TJ and Kirschner MW (1997) Expression cloning in the test tube. Science **277:** 973-974

Kionka C and Amrhein N (1984) The enzymatic malonylation of 1-aminocyclopropane-1-carboxylic acid in homogenates of mung bean hypocotyls. Planta **162:** 226-235

Klein M, Mamnun YM, Eggmann T, Schuller C, Wolfger H, Martinoia E and Kuchler K (2002) The ATP-binding cassette (ABC) transporter Bpt1p mediates vacuolar sequestration of glutathione conjugates in yeast. FEBS Letters **520:** 63-67

Klein M, Martinoia E, Hoffmann-Thoma G and Weissenbock G (2000) A membrane-potential dependent ABC-like transporter mediates the vacuolar uptake of rye flavone glucuronides: regulation of glucuronide uptake by glutathione and its conjugates. Plant Journal **21:** 289-304

Klein M, Martinoia E, Hoffmann-Thoma G and Weissenbock G (2001) The ABC-like vacuolar transporter for rye mesophyll flavone glucuronides is not species-specific. Phytochemistry **56:** 153-159

Klein M, Martinoia E and Weissenbock G (1998) Directly energized uptake of beta-estradiol 17-(beta-D-glucuronide) in plant vacuoles is strongly stimulated by glutathione conjugates. Journal of Biological Chemistry **273:** 262-270

Klein M, Weissenbock G, Dufaud A, Gaillard C, Kreuz K and Martinoia E (1996) Different energization mechanisms drive the vacuolar uptake of a flavonoid glucoside and a herbicide glucoside. Journal of Biological Chemistry **271:** 29666-29671

Koester J, Bussmann R and Barz W (1984) Malonyl-coenzyme-a - isoflavone 7-*O*-glucoside-6"-*O*- malonyltransferase from roots of chick pea (*Cicer-Arietinum*-L). Archives of Biochemistry and Biophysics **234:** 513-521

Konishi T and Sasaki Y (1994) Compartmentalization of two forms of acetyl-CoA carboxylase in plants and the origin of their tolerance towards herbicides. Proceedings of the National Academy of Sciences of the United States of America **91:** 3598-3601

Kreuz K, Tommasini R and Martinoia E (1996) Old enzymes for a new job - Herbicide detoxification in plants. Plant Physiology **111:** 349-353

Lamoureux GL and Rusness DG (1986) Tridiphane [2-(3,5-dichlorophenyl)-2-(2,2,2-trichloroethyl) oxirane] an atrazine synergist: enzymatic conversion to a potent glutathione *S*-transferase inhibitor. Pesticide Biochemistry and Physiology **26:** 323-342

Lange BM, Hertkorn N and Sandermann H (1998) Chloroaniline/lignin conjugates as model system for nonextractable pesticide residues in crop plants. Environmental Science & Technology **32:** 2113-2118

Lao SH, Loutre C, Brazier M, Coleman JO, Cole DJ, Edwards R and Theodoulou FL (2003) 3,4-Dichloroaniline is detoxified and exported via different pathways in *Arabidopsis* and soybean. Phytochemistry **63:** 653-661

Lee H and Raskin I (1999) Purification, cloning, and expression of a pathogen inducible UDP-glucose: Salicylic acid glucosyltransferase from tobacco. Journal of Biological Chemistry **274:** 36637-36642

Li S, Hartman GL and Widholm JM (1999) Viability staining of soybean suspension-cultured cells and a seedling stem cutting assay to evaluate phytotoxicity of *Fusarium solani* f. sp. glycines culture filtrates. Plant Cell Reports **18:** 375-380

Liang P and Pardee AB (1992) Differential Display of Eukaryotic Messenger-RNA by Means of the Polymerase Chain-Reaction. Science **257:** 967-971

Liu Y, Hoffman NE and Yang SF (1983) Relationship between the Malonylation of 1-Aminocyclopropane-1- Carboxylic Acid and D-Amino Acids in Mung-Bean Hypocotyls. Planta **158:** 437-441

Liu Y, Silverstone AL, Wu YM and Yang SF (1995) Formation of *N*-malonyl-L-tryptophan in water-stressed tomato leaves. Phytochemistry **40:** 691-697

Livingston AG and Willacy A (1991) Degradation of 3,4-Dichloroaniline in Synthetic and Industrially Produced Wastewaters by Mixed Cultures Freely Suspended and Immobilized in a Packed-Bed Reactor. Applied Microbiology and Biotechnology **35:** 551-557

Loutre C, Dixon DP, Brazier M, Slater M, Cole DJ and Edwards R (2003) Isolation of a glucosyltransferase from *Arabidopsis thaliana* active in the metabolism of the persistent pollutant 3,4-dichloroaniline. Plant J **34:** 485-493

Mackenbrock U, Gunia W and Barz W (1993) Accumulation and Metabolism of Medicarpin and Maackiain Malonylglucosides in Elicited Chickpea (*Cicer-Arietinum L*) Cell-Suspension Cultures. Journal of Plant Physiology **142:** 385-391

Mackenbrock U, Vogelsang R and Barz W (1992) Isoflavone and Pterocarpan Malonylglucosides and Beta-1,3- Glucan- and Chitin-Hydrolases Are Vacuolar Constituents in Chickpea (*Cicer-Arietinum* L). Zeitschrift Fur Naturforschung C-a Journal of Biosciences **47:** 815-822

Maeshima M (2001) Tonoplast transporters: Organization and function. Annual Review of Plant Physiology and Plant Molecular Biology **52:** 469-497

Marrs KA (1996) The functions and regulation of glutathione S-transferases in plants. Annual Review of Plant Physiology and Plant Molecular Biology **47:** 127-158

Martin MN and Saftner RA (1995) Purification and characterization of 1-aminocyclopropane-1-carboxylic acid *N*-malonyltransferase from tomato fruit. Plant Physiology **108:** 1241-1249

Martinez-Reina G, Matilla AJ, Martin-Remesal C, Gallardo M and Munoz De Rueda P (1996) Biochemical properties of 1-aminocyclopropane-1-carboxylate *N*-malonyltransferase activity from early growing embryonic axes of chick-pea (*Cicer arietinum* L.) seeds. Journal of Experimental Botany **47:** 1771-1778

Martinoia E, Grill E, Tommasini R, Kreuz K and Amrhein N (1993) ATP-dependent glutathione S-conjugate export pump in the vacuolar membrane of plants. Nature **364:** 247-249

Martinoia E, Massonneau A and Frangne N (2000) Transport processes of solutes across the vacuolar membrane of higher plants. Plant and Cell Physiology **41:** 1175-1186

Matern U, Feser C and Hammer D (1983) Further characterization and regulation of malonyl-coenzymeA-flavonoid glucoside malonyltransferases from parsley cell-suspension cultures. Archives of Biochemistry and Biophysics **226:** 206-217

Matern U, Feser C and Heller W (1984) *N*-Malonyltransferases from peanut. Archives of Biochemistry and Biophysics **235:** 218-227

Matern U, Reichenbach C and Heller W (1986) Efficient Uptake of Flavonoids into Parsley (*Petroselinum-Hortense*) Vacuoles Requires Acylated Glycosides. Planta **167:** 183-189

May MJ and Leaver CJ (1993) Oxidative stimulation of glutathione synthesis in *Arabidopsis- thaliana* suspension-cultures. Plant Physiology **103:** 621-627

May RG, Sparrer I, Hoque E and Sandermann H (1997) Mineralization of native pesticidal plant cell-wall complexes by the white-rot fungus, *Phanerochaete chrysosporium*. Journal of Agricultural and Food Chemistry **45:** 1911-1915

Miller GC, Zisook R and Zepp R (1980) Photolysis in 3,4-Dichloroaniline in natural waters. Journal of Agricultural and Food Chemistry **28:** 1053-1056

Monks TJ, Anders MW, Dekant W, Stevens JL, Lau SS and van Bladeren PJ (1990) Glutathione conjugate mediated toxicologies. Toxicol. Appl. Pharmacol. **106:** 1-19

Moody K, Kust CA and Buchholtz KP (1970) Uptake of herbicides by soybean roots in culture solutions. Weed Science **18:** 642-647

Noctor G and Foyer CH (1998) Ascorbate and glutathione: Keeping active oxygen under control. Annual Review of Plant Physiology and Plant Molecular Biology **49:** 249-279

Noh B, Murphy AS and Spalding EP (2001) Multidrug resistance-like genes of Arabidopsis required for auxin transport and auxin-mediated development. Plant Cell **13:** 2441-2454

Oparka KJ (1991) Uptake and Compartmentation of Fluorescent-Probes by Plant-Cells. Journal of Experimental Botany **42:** 565-579

Oswald TH, Smith AE and Phillips DV (1977) Callus and plantlet regeneration from cell cultures of Ladino clover and soybean. Physiologia Plantarum **39:** 129-134

Othmen K and Boule P (1999) Photochemical behaviour of dichloroanilines in water and formation of aminochlorophenoxazones. Journal of Photochemistry and Photobiology a-Chemistry **121:** 161-167

Parry AD, Tiller SA and Edwards R (1994) The effects of heavy metals and root immersion on isoflavonoid metabolism in alfalfa (*Medicago sativa* L.). Plant Physiology **106:** 195-202

Perrin C, Dempsey B and E.P. Serjeant (1981) pKa prediction for organic acids and bases. Chapman and Hall, London and New York

Pflugmacher S and Sandermann H (1998) Taxonomic distribution of plant glucosyltransferases acting on xenobiotics. Phytochemistry **49:** 507-511

Pilmoor JB and Gaunt JK (1981) The behaviour and mode of action of the phenoxyacetic acids in plants. *In* DH Hutson and TR Roberts, eds, Progress in Pesticide Biochemistry. John Wiley and Sons, London, pp147-218

Pueppke SG, Bolanos-Vasquez MC, Werner D, Bec-Ferte MP, Prome JC and Krishnan HB (1998) Release of flavonoids by the soybean cultivars McCall and peking and their perception as signals by the nitrogen-fixing symbiont *Sinorhizobium fredii*. Plant Physiology **117:** 599-606

Rea PA (1999) MRP subfamily ABC transporters from plants and yeast. Journal of Experimental Botany **50:** 895-913

Rea PA, Li ZS, Lu YP, Drozdowicz YM and Martinoia E (1998) From vacuolar GS-X pumps to multispecific ABC transporters. Annual Review of Plant Physiology and Plant Molecular Biology **49:** 727-760

Reverdatto S, Beilinson V and Nielson NC (1999) A multisubunit acetyl coenzyme A carboxylase from soybean. Plant Physiology **119:** 961-978

Riechers DE, Dean E, Kleinhofs A, Irzyk GP and Jones SS (1998) Chromosomal localisation and expression of a herbicide safener-regulated glutathione S-transferase gene in *Triticum aestivum* and linkage relations to *Hordeum vulgare*. Genome **41:** 368-372

Rieder G, Buchholtz KP and Kust CA (1969) Uptake of herbicides by soybean seed. Weed Science: 101-105

Robineau T, Batard Y, Nedelkina S, F. C-H, LeRet M, Sorokine O, Didierjean L and Werck-Reichhart D (1998) The chemically-inducible plant cytochrome P450 CYP76B1 actively metabolizes phenylureas and other xenobiotics. Plant Physiology **188:** 1049-1056

Ryan PR and Walker NA (1994) The Regulation of Ammonia Uptake in *Chara-Australis*. Journal of Experimental Botany **45:** 1057-1067

Sagerström CG, Sun BI and Sive HL (1997) Substractive cloning: past, present and future. Annual Review of Biochemistry **66:** 751-783

Sandermann H (1994) Higher plant metabolism of xenobiotics: the 'green liver' concept. Pharmacogenetics **4:** 225-241

Sandermann H, Hertkorn N, May RG and Lange BM (2001) Bound pesticidal residues in crop plants: chemistry, bioavailability and toxicology. *In* Pesticide Transformations in Plants and Microorganisms: Similarities and Divergences. American Chemical Society, pp 119-127

Sandermann H, Musick TJ and Aschbacher PW (1992) Animal Bioavailability of a 3,4-Dichloroaniline Lignin Metabolite Fraction from Wheat. Journal of Agricultural and Food Chemistry **40:** 2001-2007

Sandermann H, Scheel D and Vandertrenck T (1984) Use of Plant-Cell Cultures to Study the Metabolism of Environmental Chemicals. Ecotoxicology and Environmental Safety **8:** 167-182

Sandermann H, Schmitt R, Eckey H and Bauknecht T (1991) Plant biochemistry of xenobiotics - Isolation and properties of soybean *O*-glucosyl and *N*-glucosyl and *O*-malonyltransferases and *N*- malonyltransferases for

chlorinated phenols and anilines. Archives of Biochemistry and Biophysics **287:** 341-350

Sandermann HJ, Haas M, Messner B, Pflugmacher S, Schroder P and Wetzel A (1997) The role of glucosyl and malonyl conjugation in herbicide selectivity. *In* KK Hatzios, ed, Regulation of enzymatic systems detoxifying xenobiotics in plants. Kluwer Academic Publishers, pp 211-231

Sasaki Y, Konishi T and Nagano Y (1995) The compartmentation of acetyl-coenzyme A carboxylase in plants. Plant Physiology **108:** 445-449

Schäffner A, Messner B, Langebartels C and Sandermann H (2002) Genes and enzymes for *in-planta* phytoremediation of air, water and soil. Acta Biotechnol. **22:** 141-152

Scheel D, Schafer W and Sandermann H (1984) Metabolism of Pentachlorophenol in Cell-Suspension Cultures of Soybean (*Glycine-Max* L) and Wheat (*Triticum-Aestivum* L) - General Results and Isolation of Lignin Metabolites. Journal of Agricultural and Food Chemistry **32:** 1237-1241

Schenk RU and Hildebrandt AC (1972) Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Canadian Journal of Botany **50:** 199-204

Schluepmann H, Pellny T, Van Dijken A, Smeekens S and Paul M (2003) Trehalose 6-phosphate is indispensable for carbohydrate utilization and growth in *Arabidopsis thaliana*. Proc Natl Acad Sci USA **100:** 6849-6854

Schmidt B (1999) Non-extractable residues of pesticides and xenobiotics in plants - A review. Recent Res. Devel. Agricultural & Food Chem. **3:** 329-354

Schmidt B, Breuer J, Thiede B and Schuphan I (1997) Metabolism of the pesticide metabolite 4-[U-C-14] nitrophenol in *Datura stramonium* (L) cell suspension culture - A model for processing of xenobiotic carbohydrate conjugates and formation of nonextractable residues. Pesticide Biochemistry and Physiology **57:** 109-118

Schmidt B, Rivero C and Thiede B (1995) 3,4-Dichloroaniline *N*-glucosyl- and *N*-malonyltransferase activities in cell cultures and plants of soybean and wheat. Phytochemistry **39:** 81-84

Schmidt B, Rivero C, Thiede B and Schenk T (1993) Metabolism of 4-Nitrophenol in Soybean Excised Leaves and Cell- Suspension Cultures of Soybean and Wheat. Journal of Plant Physiology **141:** 641-646

Schmidt B, Thiede B and Rivero C (1994) Metabolism of the Pesticide Metabolites 4-Nitrophenol and 3,4- Dichloroaniline in Carrot (*Daucus-Carota*) Cell-Suspension Cultures. Pesticide Science **40:** 231-238

Schmitt R, Kaul J, Vondertrenck T, Schaller E and Sandermann H (1985) Beta-D-Glucosyl and O-Malonyl-Beta-D-Glucosyl Conjugates of Pentachlorophenol in Soybean and Wheat - Identification and Enzymatic-Synthesis. Pesticide Biochemistry and Physiology **24:** 77-85

Schmitt R and Sandermann H (1982) Specific localisaton of b-D-glucoside conjugates of 2,4-dichlorophenoxyacetic acid in soybean vacuoles. Zeitschrift Fur Naturforschung C-a Journal of Biosciences **37c:** 772-777

Schneider B, Schutte HR and Tewes A (1984) Comparative Investigations on the Metabolism of 2-(2,4- Dichlorophenoxy)Isobutyric Acid in Plants and Cell-Suspension Cultures of Lycopersicon-Esculentum. Plant Physiology **76:** 989-992

Schröder P (1997) Fate of glutathione S-conjugates in plants. *In* KK Hatzios, ed, Regulation of enzymatic systems detoxifying xenobiotics in plants. Kluwer Academic Publishers, The Netherlands, pp 211-231

Schröder P, Scheer C and Belford BJD (2001) Metabolism of organic xenobiotics in plants: conjugating enzymes and metabolic end points. Minerva Biotecnologica **13:** 85-91

Schuler MA (1996) Plant cytochrome P450 monooxygenases. Critical Reviews in Plant Sciences **15:** 235-284

Sheehan D, Meade G, Foley VM and Dowd CA (2001) Structure, function and evolution of glutathione transferases: implications for classification of non-mammalian members of an ancient enzyme superfamily. Biochemical Journal **360:** 1-16

Shorrosh BS, Dixon RA and Ohlrogge JB (1994) Molecular cloning, characterization and elicitation of acetyl-coenzyme a carboxylase from alfalfa. Proceedings of the National Academy of Sciences of the United States of America **91:** 4323-4327

Shukla A, Dupont S and Devine MD (1997) Resistance to ACCase-inhibitor herbicides in wild oat: Evidence for target site-based resistance in two biotypes from Canada. Pesticide Biochemistry and Physiology **57:** 147-155

Siminszky B, Corbin FT, E.R. W, Fleischmann TJ and Dewey RE (1999) Expression of a cytochrome P450 monooxygenase cDNA in yeast and tobacco enhances the metabolism of phenylurea herbicides. Proceedings of the National Academy of Sciences of the United States of America **96:** 1750-1755

Singleton SD and Murphy SD (1973) Propanil (3,4-dichloropropionalinine)-induced methemoglobin formation in mice in relation to acylamidase activity. Toxicol. Appl. Pharmacol. **25:** 20-29

Stellwagen E (1990) Chromatography on immobilized reative dyes. *In* MP Deutscher, ed, Guide to protein purification, Vol 182. Academic Press, pp 343-357

Sterling TM (1994) Mechanisms of Herbicide Absorption across Plant Membranes and Accumulation in Plant-Cells. Weed Science **42:** 263-276

Still GG (1968) Metabolism of 3,4-dichloropropionanilide in plants: the metabolic fate of the 3,4-dichloroaniline moiety. Science **159:** 992-993

Still GG, Balba HM and Mansager ER (1981) Studies on the nature and identity of bound chloroaniline residues in plants. Journal of Agricultural and Food Chemistry **29:** 739-746

St-Pierre B, Laflamme P, Alarco AM and De Luca V (1998) The terminal O-acetyltransferase involved in vindoline biosynthesis defines a new class of proteins responsible for coenzyme A-dependent acyl transfer. Plant Journal **14:** 703-713

Struijs J and Rogers JE (1989) Reductive Dehalogenation of Dichloroanilines by Anaerobic Microorganisms in Fresh and Dichlorophenol-Acclimated Pond Sediment. Applied and Environmental Microbiology **55:** 2527-2531

Surovtseva EG, Ivoilov VS, Vasileva GK and Belyaev SS (1996) Degradation of chlorinated anilines by certain representatives of the genera *Aquaspirillum* and *Paracoccus*. Microbiology **65:** 553-559

Suzuki H, Nakayama T, Yonekura-Sakakibara K, Fukui Y, Nakamura N, Nakao M, Tanaka Y, Yamaguchi M, Kusumi T and Nishino T (2001) Malonyl-CoA : anthocyanin 5-*O*-glucoside-6 "'-*O*- malonyltransferase from scarlet sage (*Salvia splendens*) flowers - Enzyme purification, gene cloning, expression, and characterization. Journal of Biological Chemistry **276:** 49013-49019

Suzuki H, Nakayama T, Yonekura-Sakakibara K, Fukui Y, Nakamura N, Yamaguchi M, Tanaka Y, Kusumi T and Nishino T (2002) cDNA cloning, heterologous expressions, and functional characterization of malonyl-coenzyme A:anthocyanidin-3-*O*-glucoside-6"-*O*-malonyltransferase from dahlia flowers. Plant Physiology **130:** 2142-2151

Suzuki T and Casida JE (1981) Metabolites of Diuron, Linuron, and Methazole Formed by Liver Microsomal-Enzymes and Spinach Plants. Journal of Agricultural and Food Chemistry **29:** 1027-1033

Theodoulou FL (2000) Plant ABC transporters. Biochimica et Biophysica Acta-Biomembranes **1465:** 79-103

Thom R, Cummins I, Dixon DP, Edwards R, Cole DJ and Lapthorn AJ (2002) Structure of a tau class glutathione *S*-transferase from wheat active in herbicide detoxification. Biochemistry **41:** 7008-7020

Thom R, Dixon DP, Edwards R, Cole DJ and Lapthorn AJ (2001) The structure of a zeta class glutathione *S*-transferase from *Arabidopsis thaliana*: Characterisation of a GST with novel active-site architecture and a putative role in tyrosine catabolism. Journal of Molecular Biology **308:** 949-962

Thomas C, Rajagopal A, Windsor B, Dudler R, Lloyd A and Roux SJ (2000) A role for ectophosphatase in xenobiotic resistance. The Plant Cell **12:** 519-533

Thomas SG, Phillips AL and Hedden P (1999) Molecular cloning and functional expression of gibberellin 2-oxidases, multifunctional enzymes involved in gibberellin deactivation. Proc Natl Acad Sci U S A **96:** 4698-4703

Thorn KA, Pettigrew PJ and Goldenberg WS (1996) Covalent binding of aniline to humic substances. 2. N-15 NMR studies of nucleophilic addition reactions. Environmental Science & Technology **30:** 2764-2775

Tiller SA, Parry AD and Edwards R (1994) Changes in the Accumulation of Flavonoid and Isoflavonoid Conjugates Associated with Plant-Age and Nodulation in Alfalfa (*Medicago-Sativa*). Physiologia Plantarum **91:** 27-36

Travkin V, Baskunov BP, Golovlev EL, Boersma MG, Boeren S, Vervoort J, van Berkel WJH, Rietjens I and Golovleva LA (2002) Reductive deamination as a new step in the anaerobic microbial degradation of halogenated anilines. FEMS Microbiology Letters **209:** 307-312

Unligil UM and Rini JM (2000) Glycosyltransferase structure and mechanism. Current Opinion in Structural Biology **10:** 510-517

Ünlü M, Morgan ME and Minden JSW (1997) Difference gel electrophoresis: A single gel method for detecting changes in protein extracts. Electrophoresis **18:** 2071-2077

Uotila M, Gullner G and Komives T (1995) Induction of glutathione *S*-transferase activity and glutathione level in plants exposed to glyphosate. Physiologia Plantarum **93:** 689-694

Valentovic MA, Ball JG, Anestis DK and Rankin GO (1995) Comparison of the *in-Vitro* Toxicity of Dichloroaniline Structural Isomers. Toxicology in Vitro **9:** 75-81

van den Brule S, Muller A, Fleming AJ and Smart CC (2002) The ABC transporter SpTUR2 confers resistance to the antifungal diterpene sclareol. Plant Journal **30:** 649-662

Vrielink A, Ruger W, Driessen HPC and Freemont PS (1994) Crystal-Structure of the DNA Modifying Enzyme Beta-Glucosyltransferase in the Presence and Absence of the Substrate Uridine Diphosphoglucose. EMBO Journal **13:** 3413-3422

Walker JE, Saraste M, Runswick MJ and Gay NJ (1982) Distantly related sequences in alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO Journal **1:** 945-951

Werck-Reichhart D, Hehn A and Didierjean L (2000) Cytochromes P450 for engineering herbicide tolerance. Trends in Plant Science **5:** 116-123

Werner C and Matile P (1985) Accumulation of coumarylglucosides in vacuoles of barley mesophyll protoplasts. Journal of Plant Physiology **118:** 237-249

Wink M (1997) Compartmentation of secondary metabolites and xenobiotics in plant vacuoles. *In* Advances in Botanical Research Incorporating Advances in Plant Pathology, Vol 25, Vol 25. Academic Press Ltd, London, pp 141-169

Winkel-Shirley B (1999) Evidence for enzyme complexes in the phenylpropanoid and flavonoid pathways. Physiologia Plantarum **107:** 142-149

Winkler R and Sandermann H (1989) Plant-Metabolism of Chlorinated Anilines - Isolation and Identification of *N*-Glucosyl and *N*-Malonyl Conjugates. Pesticide Biochemistry and Physiology **33:** 239-248

Winkler R and Sandermann H (1992) *N*-Glucosyl conjugates of chlorinated anilines - Spontaneous formation and cleavage. Journal of Agricultural and Food Chemistry **40:** 2008-2012

Wu YM, Silverstone AL, Liu Y and Yang SF (1995) Partial-purification and characterization of tryptophan *N*-malonyltransferase from tomato leaves. Phytochemistry **40:** 699-704

Yang SF and Hoffman NE (1984) Ethylene Biosynthesis and Its Regulation in Higher-Plants. Annual Review of Plant Physiology and Plant Molecular Biology **35:** 155-189

Yih RY, McRae DH and Wilson HF (1968) Metabolism of 3',4'-dichloropropionanilide: 3,4-dichloroaniline-lignin complex in rice plants. Science **161:** 376-377

APPENDICES

APPENDIX 1

3,4-Dichloroaniline is detoxified and exported via different pathways in *Arabidopsis* and soybean

S. Lao *et al.*, 2003. Phytochemistry 63, 653-661

Available online at www.sciencedirect.com
SCIENCE DIRECT®
PERGAMON
Phytochemistry 63 (2003) 653–661
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

3,4-Dichloroaniline is detoxified and exported via different pathways in *Arabidopsis* and soybean

Si-Houy Lao[a], Caroline Loutre[b], Melissa Brazier[b], Julian O.D. Coleman[c], David J. Cole[d], Robert Edwards[b], Frederica L. Theodoulou[a,*]

[a]*Crop Performance and Improvement Division, Rothamsted Research, Harpenden AL5 2JQ, UK*
[b]*School of Biological and Biomedical Sciences, University of Durham, Durham DH1 3LE, UK*
[c]*School of Biological and Molecular Sciences, Oxford Brookes University, Gypsy Lane, Oxford OX3 0BP, UK*
[d]*Formerly of Aventis Crop Science, Fyfield Road, Ongar, Essex CM5 0HW, UK*

Received 4 February 2003; received in revised form 28 April 2003

Abstract

The metabolic fate of [UL-^{14}C]-3,4-dichloroaniline (DCA) was investigated in *Arabidopsis* root cultures and soybean plants over a 48 h period following treatment via the root media. DCA was rapidly taken up by both species and metabolised, predominantly to *N*-malonyl-DCA in soybean and *N*-glucosyl-DCA in *Arabidopsis*. Synthesis occurred in the roots and the respective conjugates were largely exported into the culture medium, a smaller proportion being retained within the plant tissue. Once conjugated, the DCA metabolites in the medium were not then readily taken up by roots of either species. The difference in the routes of DCA detoxification in the two plants could be explained partly by the relative activities of the respective conjugating enzymes, soybean containing high DCA-*N*-malonyltransferase activity, while in *Arabidopsis* DCA-*N*-glucosyltransferase activity predominated. A pre-treatment of plants with DCA increased DCA-*N*-malonyltransferase activity in soybean but not in *Arabidopsis*, indicating differential regulation of this enzyme in the two plant species. This study demonstrates that DCA can undergo two distinct detoxification mechanisms which both lead to the export of conjugated metabolites from roots into the surrounding medium in contrast to the vacuolar deposition more commonly associated with the metabolism of xenobiotics in plants.

Keywords: Glycine max (Fabaceae) soybean; *Arabidopsis thaliana* (Brassicaceae); Herbicide metabolism; Conjugation; Export; Detoxification; Dichloroaniline; Malonyltransferase; Glucosyltransferase; Roots

1. Introduction

The ability of plants to take up and chemically transform xenobiotics has important implications for environmental and food science. Chlorinated anilines such as 3,4-dichloroaniline (DCA) are intermediates in chemical synthesis as well as being metabolites of acylanilide, phenylurea, and carbamate pesticides (Parris, 1980; Van der Trenck et al., 1981). DCA can persist in the environment as insoluble residues in soil and plants and can also photodimerise to form carcinogens (Harvey et al., 2002). Consequently, DCA has been classified as a compound of environmental concern, with substantial interest in its metabolic fate in plants (Bartha et al., 1983; Sandermann et al., 1983; Harvey et al., 2002). DCA metabolism has been investigated in a small number of plants, with differences observed in the proportions and identities of biotransformation products. In soybean, *N*-malonylation was the major route of DCA metabolism in cell cultures (Winkler and Sandermann, 1989; Harms and Langbartels, 1986; Gareis et al., 1992; Schmidt et al., 1995), excised leaves (Gareis et al., 1992) and whole plants (Bockers et al., 1994). In wheat, both plants and suspension-cultured cells metabolised DCA mainly by *N*-glucosylation (Schmitt et al., 1985, 1995; Winkler and Sandermann, 1989, 1995; Bockers et al., 1994), while in carrot cultures, DCA underwent both malonylation and glucosylation (Schmidt et al., 1994). The enzymes responsible for catalysing the conjugation of DCA to glucose and malonic

* Corresponding author. Fax: +44-1582-763010.
E-mail address: freddie.theodoulou@bbsrc.ac.uk (F.L. Theodoulou).

0031-9422/03/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/S0031-9422(03)00289-9

acid respectively have been described in plants (Frear, 1968; Sandermann et al., 1991; Matern et al., 1984). In a number of studies, species-dependent differences in DCA metabolism have been attributed to differences in the activity of the respective DCA-*N*-glucosyltransferase and DCA-*N*-malonyltransferase enzymes (Sandermann et al., 1991; Schmidt et al., 1995).

In this study, we have compared DCA metabolism in soybean, a species where the detoxification of this xenobiotic is relatively well characterised, with its metabolism in the model species, *Arabidopsis thaliana*. Ultimately, the intention here is to characterise the enzymes and transporters involved in DCA metabolism in these two species using a combination of biochemical and molecular approaches, with the potential aim of manipulating xenobiotic metabolism in plants.

2. Results

2.1. Time course of ^{14}C DCA uptake

Hydroponically-grown soybean seedlings and *Arabidopsis* root cultures were treated with identical, sub-toxic doses of [UL-^{14}C]-3,4-dichloroaniline (45.2 nmol, 37 kBq) for 48 h to investigate the uptake and distribution of radioactivity over time. Plants were extracted with methanol to determine incorporation into soluble metabolites, with any non-extractable radioactivity defined as being a bound residue which was quantified after digestion with alkali. Radioactivity in the medium was quantified directly, with the aqueous medium partitioned with ethyl acetate to recover DCA and its metabolites (average recovery 86% in soybean and 96% in *Arabidopsis*). In each case, the radioactivity present was quantified by liquid scintillation counting (LSC; Fig. 1). In soybean plants, radioactivity in the medium declined rapidly over 0–4 h (Fig. 1A). A major proportion of the loss in radioactivity was due to the uptake of [^{14}C]-DCA into the soybean plants, where the radiolabel accumulated as both extractable and bound residues, predominantly in the roots. After 4 h, the distribution of radiolabel between medium and root remained constant. There was a moderate net loss of radioactivity from the system after 48 h, which was attributed to the volatilisation of [^{14}C]-DCA from the medium due to aeration. Fluorographs of the [^{14}C]-DCA treated soybean plants showed that, at the end of the time course, the majority of the label had accumulated in the roots, with a small proportion in the meristem (Fig. 2).

In *Arabidopsis*, the rate of net uptake of radioactivity into cultured roots was also rapid, with soluble residues in the roots comprising 65% of total label after 24 h of treatment (Fig. 1B). Unlike soybean, only 3–4% of the administered label was incorporated into bound residues in *Arabidopsis* roots. The percentage of the dosed radiolabel in the *Arabidopsis* growth medium continued to decline during the course of the experiment, to a final value of 20% after 48 h treatment.

2.2. Metabolic fate of DCA in soybean and Arabidopsis

The radioactive metabolites present in the plant tissues and respective media were analysed by thin layer chromatography (TLC) and quantified, following Phosphorimaging. For reference, the profiles of metabolites seen in both soybean and *Arabidopsis* after a 48 h incubation with [^{14}C]-DCA are shown in Fig. 3. The identification of DCA metabolites was based on their co-chromatography in two TLC solvent systems and by HPLC (data not shown) with prepared standards of DCA-*N*-malonate (M-DCA) and [^{14}C]-DCA-*N*-β-D-glucoside (G-DCA), which are both well characterised metabolites of DCA in plants (Winkler and Sandermann, 1989, 1992; Gareis et al., 1992).

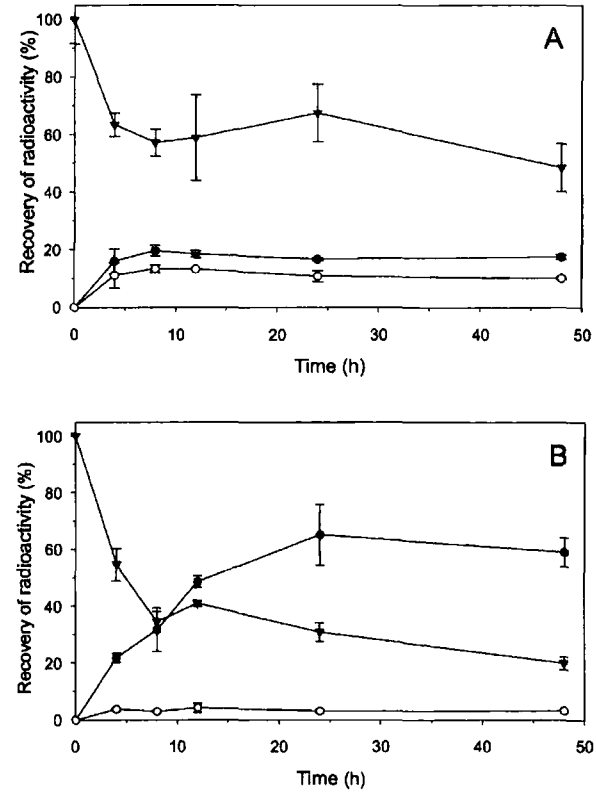


Fig. 1. Distribution of radioactivity following addition of [^{14}C]-DCA to the root medium of soybean and *Arabidopsis*. Hydroponically-grown soybean seedlings (A), and *Arabidopsis* root cultures (B) were treated with 45.2 nmol (37 kBq) [UL-^{14}C]-3,4-dichloroaniline for 48 h. At various time points, the radioactive metabolites were extracted from the plants and medium and quantified by LSC as: soluble residues in the plant (closed circles), bound residues in the plant (open circles), radioactivity in the growth medium (closed triangles). Data points represent the means of 2–4 replicates, with error bars showing standard deviations. Representative of four experiments.

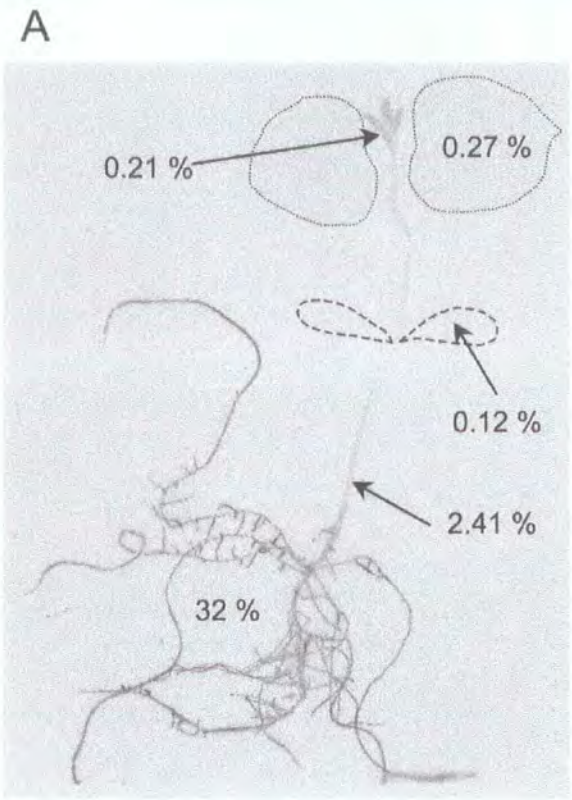


Fig. 2. Distribution of radiolabel in soybean plant fed [^{14}C]-DCA through the root medium. Hydroponically-grown, 10 day-old soybean seedlings were treated with 45.2 nmol (37 kBq) [UL-^{14}C]-3,4-dichloroaniline. After 3.5 days, plants were analysed by phosphor-imaging (A). Radioactivity in the dissected plant parts was quantified by LSC and the results expressed as % of the original dose (A) and nmol of [^{14}C]-DCA equivalents g^{-1} FW (B), with results showing the means of duplicated experiments±S.E.

In all chromatograms, the parent [^{14}C]-DCA ran as two radioactive entities, with the proportion of radioactivity in each band determined by the solvent system. In the chloroform:methanol:water system, 92% of the radioactivity in the [^{14}C]-DCA dosing solution co-chromatographed with authentic unlabelled DCA, while the

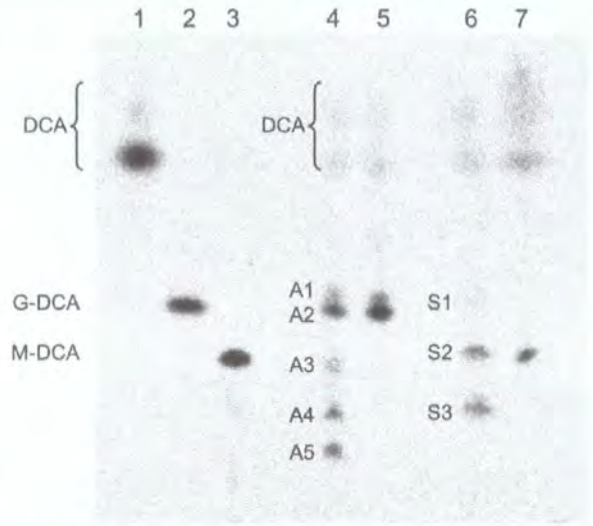


Fig. 3. Identification of DCA metabolites by TLC. Radioactive DCA metabolites were extracted from plants and culture medium and analysed by TLC using chloroform/methanol/water (60:35:8; v/v/v). Lanes: (1) [^{14}C]-3,4-dichloroaniline, (2) *N*-^{14}C-D-glucopyranosyl)-3,4-dichloroaniline, (3) *N*-^{14}C-malonyl-3,4-dichloroaniline, (4) *Arabidopsis* plant soluble extract, (5) *Arabidopsis* growth medium, (6) soybean plant soluble extract, (7) soybean growth medium.

other 8% ran as a diffuse spot near the solvent front. When the "authentic" [^{14}C]-DCA was scraped off and re-run in the same chromatography system, the radioactivity again partitioned into the two moieties in a similar ratio (9:1). It was concluded that the minor, more hydrophobic radioactive entity was an artefact of chromatography, rather than an impurity and in all subsequent analyses, these two radioactive bands were collectively quantified as parent [^{14}C]-DCA. When analysed by HPLC, [^{14}C]-DCA ran as a single radioactive entity (data not shown).

Previous studies have demonstrated that DCA can spontaneously form the respective *N*-β-D-glucopyranosyl conjugates when incubated with D-glucose (Winkler and Sandermann, 1992). It was important, therefore, to demonstrate that DCA metabolism in the root cultures was due to reactions mediated in planta, rather than due to non-enzymic reactions. When [^{14}C]-DCA was incubated with *Arabidopsis* growth medium containing 1% (w/v) D-glucose in place of sucrose, G-DCA was seen to accumulate at a rate of 0.15 nmol h^{-1} under the dosing conditions used in the plant feeding studies. When sucrose, the sugar used routinely in the plant studies was tested, [^{14}C]-DCA was not converted to polar conjugates. Similarly, the rate of DCA conjugate synthesis in conditioned media taken from the *Arabidopsis* root cultures or soybean plants was also below the limit of detection. These experiments confirmed that any metabolism of DCA observed in the feeding studies was due to metabolism in planta.

After a 48 h incubation with [^{14}C]-DCA, distinct profiles of radioactive metabolites were seen in soybean and *Arabidopsis* (Fig. 3). Extracts from soybean plants contained three major metabolites of DCA which were designated S1, S2 and S3, respectively. Based on co-chromatography, S1 was identified as G-DCA and S2 as M-DCA. Compound S3 did not co-chromatograph with any available standards. In the soybean root medium, only S2 (M-DCA) was observed in addition to [^{14}C]-DCA. Extracts from *Arabidopsis* plants contained five major metabolites of DCA, designated A1–A5. Compound A1 was not identified but migrated very closely to A2, which in turn co-chromatographed with G-DCA. Metabolite A3 appeared to be M-DCA while A4 was identical to the unknown soybean DCA metabolite S3. The polar compound A5 was also not characterised. Although metabolites A1, A4 and A5 were not identified, their lability to acid hydrolysis and associated release of DCA suggested that they were all *N*-conjugates of the parent compound. In the *Arabidopsis* root medium, only A1 and G-DCA were observed as DCA metabolites.

The metabolism of [^{14}C]-DCA in soybean plants and *Arabidopsis* root cultures was studied over a 48 h feeding period and the major recovered metabolites (Fig. 3) quantified in both media and plant tissue (Table 1). With soybean, parent [^{14}C]-DCA steadily declined from the medium, to be replaced with M-DCA. In contrast, the M-DCA content in the soybean plant remained virtually constant throughout this period. By comparison with the proportion of DCA metabolised by malonylation, the amounts of G-DCA and metabolite S3 formed were modest, with G-DCA accumulating transiently at 2 h and then declining.

In *Arabidopsis*, G-DCA was the dominant metabolite at all time points in both the roots and the media. In contrast, M-DCA remained a minor metabolite, confirming that glucosylation was the major route of DCA conjugation in *Arabidopsis*. In the roots, the accumulation of G-DCA was particularly marked over the first 4 h, after which time its relative abundance steadily declined. During this loss of G-DCA, the amounts of DCA and M-DCA remained constant, A4 and A5 slowly accumulated and A1 slowly declined. The selective disappearance of G-DCA from the roots was associated with an increase in the glucoside in the medium, suggesting that the conjugate was being exported. Similarly, the slower decline in A1 in the roots was mirrored by its steady accumulation in the medium.

Table 1
Quantification of major DCA metabolites formed over 48 h in soybean plants and *Arabidopsis* root cultures dosed with 45.2 nmol [^{14}C]-DCA[a]

Time (h)	Extracted metabolites (nmol)					
	DCA	A1	G-DCA	M-DCA	A4/S3	A5
Soybean root extract						
0.5	1.5		ND	0.6	0.1	
2	4.4		0.4	2.2	0.4	
4	4.2		0.3	1.5	0.4	
8	5.5		ND	0.6	0.5	
12	5.0		ND	0.6	0.2	
24	4.2		ND	0.9	0.2	
48	3.8		ND	1.3	0.2	
Soybean medium						
0.5	29.5			2.0		
2	15.8			4.7		
4	7.8			7.0		
8	4.1			11.3		
12	4.4			11.7		
24	3.3			15.1		
48	3.2			12.0		
Arabidopsis root extract						
4	2.7	3.1	18.2	0.5	0.9	0.5
8	2.2	2.5	9.7	0.3	0.9	0.5
12	3.4	2.5	10.9	0.5	1.4	0.6
24	2.2	2.0	6.5	0.6	1.7	1.4
48	1.3	0.9	3.0	0.8	2.5	2.0
Arabidopsis medium						
4	2.7	0.3	6.1	ND		
8	3.0	2.2	9.5	ND		
12	5.6	3.0	12.8	1.1		
24	2.2	5.8	22.0	0.5		
48	2.8	6.7	17.5	0.6		

[a] Results represent the means of duplicated studies with the variation in the plants being < 10% of the mean. ND = not detected.

2.3. Transferase activities

In order to account for the differences in DCA conjugation in soybean and *Arabidopsis*, the activities of 3,4-dichloroaniline-*N*-malonyltransferase (DCA-*N*-MT) and 3,4-dichloroaniline-*N*-glucosyltransferase (DCA-*N*-GT) were determined in crude root extracts from the two species. In both cases, product formation was monitored by quantifying the amount of radiolabelled M-DCA or G-DCA formed which partitioned into ethyl acetate (Brazier et al., 2002). With both assays, the activities reported significantly underestimate the true catalytic efficiencies of the respective enzymes, as in both cases they were incubated with low concentrations of the radiolabelled co-substrate, either UDP-[^{14}C-glucose] or [^{14}C-malonyl]-CoA. This was done to enhance the sensitivity of the assays by keeping the radioactive specific activities of the substrates high. However, with both DCA-*N*-MT and DCA-*N*-GT activities, it was demonstrated that product formation was strictly dependent on protein content for all comparative assays performed. In soybean roots, DCA-*N*-MT activity was high (12 nkat g^{-1} protein), whereas DCA-*N*-GT was barely detectable (0.5 pkat g^{-1} protein). In *Arabidopsis* root cultures, DCA-*N*-GT and DCA-*N*-MT activities were readily detectable although DCA-*N*-GT activity was two orders of magnitude greater than DCA-*N*-MT (6.3 nkat g^{-1} protein and 52 pkat g^{-1} protein, respectively). No DCA-*N*-MT or

DCA-*N*-GT activity could be determined in the media immersing either the soybean or *Arabidopsis* roots, confirming that the synthesis of the respective conjugates occurred within the plant tissues.

Since many xenobiotic-metabolising enzymes have been shown to be inducible (Marrs, 1996; Tommasini et al., 1997; Robineau et al., 1998), the effects of DCA pre-treatment on DCA-*N*-MT and DCA-*N*-GT activity (assayed at saturating DCA) were determined. Pre-treatment of *Arabidopsis* root cultures with 100 μM DCA for up to 48 h had no effect on DCA-*N*-MT or DCA-*N*-GT activity (data not shown). In soybean roots however, DCA-*N*-MT activity was increased by 2- to 6-fold depending on the length of treatment with 100 μM DCA (Fig. 4A). Increasing the DCA concentration to 200 μM gave no further enhancement in activity compared to 100 μM pre-treatment. Kinetic analysis of the effect of DCA pre-treatment indicated that although V_{maxDCA} was somewhat variable between experiments (control: 248.83±272.40 nkat g^{-1}; DCA-treated: 1376±474 nkat g^{-1}), this parameter was always increased by DCA pre-treatment (fig. 4B). In contrast, K_{mDCA} did not change substantially (control: 23.09±3.17 μM; DCA-treated: 30.75±5.15 μM).

2.4. Cross-feeding study

Since metabolic fate studies indicated that DCA was exported from roots in a species-specific manner, predominantly as M-DCA in soybean and G-DCA in *Arabidopsis*, a cross-feeding experiment was conducted to determine whether or not these metabolites could be reabsorbed by either plant. Soybean seedlings and *Arabidopsis* root cultures were grown in the presence of 37 kBq [UL-^{14}C]-DCA (45.2 nmol). After 48 h, the metabolites released into the medium by each plant species were extracted with ethyl acetate, concentrated and quantified by LSC. Untreated plants from both species were then incubated for 24 h in 20 ml fresh medium containing 6.67 kBq ^{14}C-labelled metabolites from each species and plants and media analysed by LSC to determine the partitioning of ^{14}C between the growth medium and plant material.

Soybean seedlings took up approximately equal amounts of ^{14}C-labelled compounds whether they were derived from soybean or *Arabidopsis* growth medium. In each case, 12% of the supplied radioactivity was recovered in the extractable fraction from the plants while 5% became incorporated into bound residues, regardless of source of the metabolites. *Arabidopsis* root cultures showed a slight preference for uptake of radiolabelled compounds derived from *Arabidopsis* culture medium: the soluble fraction recovered from plants corresponded to 13% of the dosed radioactivity when *Arabidopsis* metabolites were supplied, compared with only 5% for soybean. Almost no bound residues were formed in *Arabidopsis*, in agreement with the DCA feeding studies (Fig. 1). After 48 h of DCA treatment, about 5–10% of the ^{14}C in the medium is unchanged DCA (see Table 1) and it is possible that the small amount of uptake and incorporation in the cross-feeding study was due to the presence of this parent compound. In both soybean and *Arabidopsis*, the majority of the radioactivity (76–93%) remained in the medium during the cross-feeding experiment, irrespective of the source of the labelled metabolites, suggesting that the export of M-DCA or G-DCA constitutes an effective mechanism of exclusion which extends across the species.

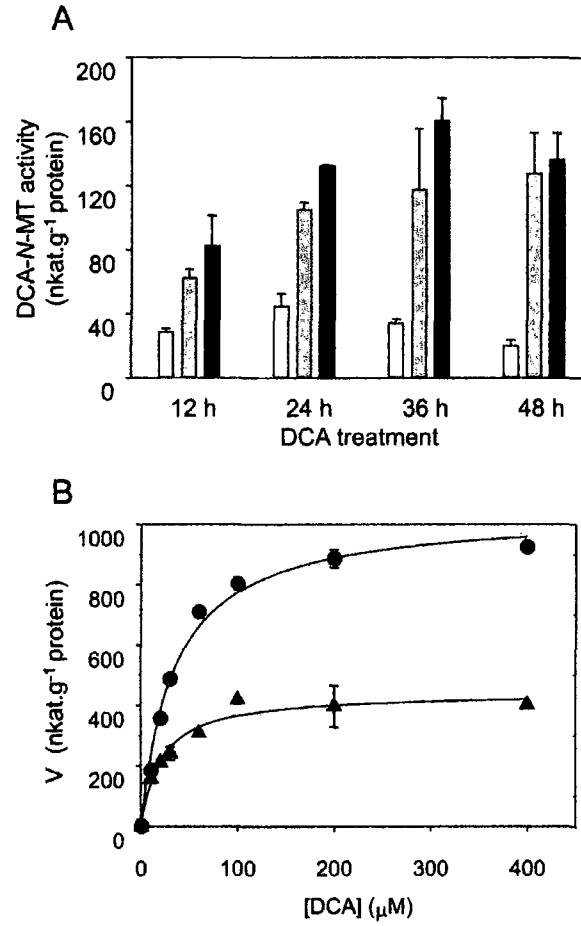


Fig. 4. Effect of DCA treatment on DCA-*N*-MT activity. (A) Hydroponically-grown soybean plants were treated with 100 μM DCA (light grey bars) or 200 μM DCA (black bars) for 12–48 h. Roots were harvested at 10 days and DCA-*N*-MT assayed. Open bars represent controls (methanol treatment). (B). Soybean roots were treated with 100 μM DCA (circles) or an equal vol. of methanol (triangles) for 24 h, harvested at 10 days and DCA-*N*-MT activities were then determined for a range of concentrations of DCA with each replicated four times (means±S.E.). K_{mDCA} and V_{maxDCA} were determined by calculating the least-squares fit to a Michaelis–Menten function. In this experiment, K_{mDCA} values were 20.84±4.04 μM and 34.39±3.91 μM in control and DCA-treated plants, respectively. V_{maxDCA} values were 441.45±21.73 nkat g^{-1} and 1040.75±35.1 nkat g^{-1} in control and DCA-treated plants, respectively.

3. Conclusions

Our results demonstrate that *N*-malonylation of DCA is the major route of detoxification in soybean, while in *Arabidopsis* *N*-glucosylation predominates. In both cases, the respective conjugates were rapidly exported from their site of synthesis in the roots into the surrounding medium. The *N*-malonylation of DCA in soybean and carrot has been reported previously (Winkler and Sandermann, 1989; Gareis et al., 1992; Bockers et al., 1994; Schmidt et al., 1994, 1995). In contrast, *N*-glucosylation is a minor route of metabolism of DCA in soybean, but a major route in monocots such as wheat and rice (Still, 1968; Schmitt et al., 1985; Winkler and Sandermann, 1989; Schmidt et al., 1995; Bockers et al., 1994). Interestingly the *N*-malonylation of DCA in soybean was linked with insoluble residue formation (Gareis et al., 1992; Bockers et al., 1994). The *N*-malonylation of DCA in soybean can be readily explained by the presence of a highly active DCA-*N*-MT in the roots with the small proportion of G-DCA formed reflecting the very low activities of DCA-*N*-GT. In earlier studies, the 6″-*O*-malonate of G-DCA was reported to be a significant metabolite of DCA in soybean (Gareis et al., 1992; Bockers et al., 1994). Attempts to demonstrate the presence of this metabolite proved inconclusive, although its chromatographic properties resemble those of metabolite S3 (=A4).

In *Arabidopsis*, the major metabolite was identified as G-DCA. In addition to G-DCA and M-DCA, three other polar, radiolabelled metabolites could be detected, all of which appear to be *N*-conjugates. In terms of abundance, compound A1 was the second most significant metabolite, accumulating along with G-DCA in the medium over time. The presence of a small amount of M-DCA in *Arabidopsis* was presumably due to the low, but measurable DCA-*N*-MT activity present, which was 100-fold less active than the DCA-*N*-GT.

Induction experiments revealed that DCA-*N*-MT is differentially regulated in *Arabidopsis* and soybean. *Arabidopsis* root cultures exhibited a low, constitutive activity which was not elevated by DCA pre-treatment, whereas DCA-*N*-MT was increased in soybean roots following pre-incubation of roots in 100 μM DCA. The DCA pre-treatment of soybean roots resulted in an increase in V_{maxDCA} for the DCA-*N*-MT, but only a modest change in K_{mDCA}, suggesting that pre-treatment increases the abundance of the enzyme present either by increasing synthesis or preventing protein turnover. This could provide a potential route to the molecular identification of DCA-*N*-MT.

In both soybean and *Arabidopsis*, a major sink of DCA metabolism involved export of the M-DCA and G-DCA conjugates into the respective media. In soybean, the export of M-DCA into the media has previously been reported to be a major route of DCA metabolism in both suspension cultured cells (Winkler and Sandermann, 1989) and the roots of whole plants (Bockers et al., 1994). Significantly, we have also demonstrated that once exported, the M-DCA was not readily re-imported into the roots of soybean or *Arabidopsis*. Since M-DCA is chemically stable, its export would therefore represent an effective route and long-term mechanism for detoxification (Winkler and Sandermann, 1989, 1992). Extracellular export of M-DCA may result from the inability of soybean to deacylate this metabolite (Matern et al., 1984). The insecticide, phoxim [*O*,*O*-diethyl α-cyanobenzylideneamino-oxyphosphononthioate; 2-(diethoxyphosphinothioloxyimino)-2-phenylacetonitrile] is also exported from soybean suspension culture cells as its *N*-malonyl conjugate (Höhl and Barz, 1995), suggesting that plasma membrane transport of *N*-malonylated xenobiotics is a general detoxification mechanism for synthetic amines. This is in contrast to the *N*-malonates of natural products such as the *N*-malonyl-conjugate of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC), which is selectively transported into the vacuole (Yang and Hoffman, 1984). In *Arabidopsis*, the small amount of M-DCA formed was also exported, though this was negligible compared to the efflux of G-DCA (Table 1). This was in contrast to wheat, with neither G-DCA nor other related glycosides being exported from the root (Harms and Langbartels, 1986; Bockers et al., 1994). Neither soybean nor *Arabidopsis* roots supported an appreciable net uptake of the *N*-glucosyl conjugate when it was supplied to hydroponic growth medium. However, unlike the *N*-malonate, G-DCA is labile to both non-enzymic and microbial hydrolysis (Winkler and Sandermann, 1992), suggesting that under field conditions the DCA would be released in the rhizosphere and become available for uptake by plants.

Malonylation and glucosylation of natural products are well known to influence the transport and storage of natural products. Thus *O*-malonylated glucosides of natural products such as anthocyanins and the isoflavone phytoalexin medicarpin, as well as *O*-malonates of chlorophyll degradation products, are compartmentalised within the vacuole (Harborne and Self, 1987; Mackenbrock et al., 1993; Barz and Mackenbrock, 1994; Hinder et al., 1996; Tommasini et al., 1998; Lu et al., 1998). It has been proposed that the malonyl group not only protects the β-glucosyl group from attack by β-glucosidases but also acts as a signal for vacuolar sequestration (Matern et al., 1983; Köster et al., 1984; Schmitt et al., 1985; Sandermann, 1994). In all these examples, *O*-malonylation can be considered to be a freely reversible modification through the action of malonylesterases located in the cytoplasm or the tonoplast (Matern, 1983; Mackenbrock et al., 1992). In contrast, *N*-malonates of xenobiotics are recalcitrant to

hydrolysis and appear to be more effectively detoxified by extracellular deposition. It also appears that *N*-glycosides of DCA are readily exported from the roots of *Arabidopsis* plants, but not from other species such as wheat. These observations raise some interesting questions about the specificity and regulation of *N*-conjugate transporters in plants and understanding these processes could be an important tool in manipulating xenobiotic metabolism in plants in the future. To that end, future work will concentrate on identifying the genes encoding the proteins responsible for the conjugation and transport of xenobiotics in *Arabidopsis* and other plants.

4. Experimental

4.1. Chemicals

3,4-Dichloroaniline (DCA) was obtained from Riedel-de-Haën (Germany) and unlabelled malonic acid from Fluka. [UL-^{14}C]-3,4-Dichloroaniline (817.7 MBq/mmol) was purchased from Sigma-Aldrich (UK) and [2-^{14}C-Malonyl]-Coenzyme A (2.035 GBq/mmol) and Uridine diphospho-D-[UL-^{14}C]-glucose (11.84 GBq/mmol) from Amersham Pharmacia Biotech (UK). *N*-malonyl-3,4-dichloroaniline (M-DCA) was prepared by chemical synthesis (Matern et al., 1984).

4.2. Feeding studies with [^{14}C]-DCA

A. thaliana, ecotype Col 0 seeds (Lehle Seeds, TX USA) were sterilised in 70% (v/v) ethanol for 1 min followed by sodium hypochlorite (20% v/v) for 10 min. After rinsing with sterile distilled water, 10 seeds were transferred to 50 ml Gamborg B5 liquid medium (Sigma-Aldrich, UK), pH 5.8, supplemented with 1% (w/v) sucrose. After growing for 17 days in the dark with constant shaking, 37 kBq (45.2 nmol) [^{14}C]-DCA was added in 50 μl methanol to each flask. At timed intervals, the root cultures and medium were separated by vacuum filtration and frozen in liquid nitrogen prior to storage at −80 °C.

Soybean seeds (*Glycine max* L. var. Chapman) were sown in vermiculite and grown at 24 °C, with 110 μE m^{-1} s^{-1} light intensity (16 h photoperiod), for 10 days. For DCA metabolism studies, the seedlings were transferred to Erlenmeyer flasks, each containing 50 ml Hoagland's No. 2 basal salt mixture (Sigma-Aldrich, UK), pH 6.0 and incubated for 24 h with constant aeration. [UL-^{14}C]-3,4-dichloroaniline (37 kBq; 45.2 nmol) was added to the media (50 ml), and the seedlings incubated with constant aeration and illumination. Duplicate batches, each of three seedlings, were harvested at timed intervals, the roots were rinsed with distilled water, blotted dry and plants separated into roots, cotyledons, primary leaves, secondary leaves, and stem. Samples were weighed, frozen in liquid nitrogen and stored at −80 °C.

4.3. Extraction of radioactive metabolites

Frozen plant tissue was ground in a pestle and mortar with acid-washed sand in the presence of ice-cold methanol (10 v/w). After centrifugation (3000 *g*, 5 min), the supernatant was decanted and the pellet frozen at −20 °C. The volume of the supernatant was noted and a sample assayed by liquid scintillation counting (LSC). The extract was then concentrated under reduced pressure at 50 °C and the residue resuspended in methanol (1 ml) and re-assayed by LSC. Non-extractable radioactivity in the plant material was determined after extensively washing the pellet from the initial extraction with methanol. The pellet was then incubated in 2 ml 2 M NaOH, at 37 °C for 20 h prior to the addition of 320 μl concentrated HCl. Samples (100 μl) were then analysed by LSC. Growth medium was made up to a known volume with water and 200 μl analysed by μLSC. The medium was then partitioned two times against one v/v ethyl acetate and the organic phases combined and concentrated to 1 ml under vacuum, at 40 °C. The residue was redissolved in 1 ml methanol and re-assayed by LSC.

4.4. Cross feeding study

Four soybean seedlings and four *A. thaliana* root cultures were transferred into 50 ml Hoagland's medium and Gamborg B5 basal salt medium respectively, each containing 37 kBq [UL-^{14}C]-DCA. After 48 h, plants were removed and the radiolabelled metabolites present in the growth media were assayed by LSC and then extracted by partitioning with 1 v/v ethyl acetate. The organic phase was evaporated under vacuum and the residue redissolved in 1 ml of methanol and assayed by LSC. After concentrating the methanolic extract to 50 μl under a stream of N_2, the equivalent of 6.67 kBq of ^{14}C-labelled metabolites was added to 20 ml of fresh media in the following combinations: (1) fresh soybean plants treated with DCA metabolites from soybean (2) fresh *Arabidopsis* root cultures treated with DCA metabolites from soybean (3) fresh soybean plants treated with DCA metabolites from *Arabidopsis* (4) fresh *Arabidopsis* root cultures treated with DCA metabolites from *Arabidopsis*. After 24 h, the plant material and growth media were radioassayed as described above.

4.5. Identification of metabolites

Samples were applied onto silica gel TLC plates precoated with fluorescent indicator (UV_{254}, Sigma-Aldrich, UK). The compounds were separated in chloroform/methanol/water (60:35:8; v/v/v) or ethyl

acetate/propan-2-ol/water (63:23:11; v/v/v). UV-absorbing spots were detected under UV light at 254 nm and 366 nm, respectively. Radioactive metabolites were detected by autoradiography using BioMax MR Film in combination with BioMax TranScreen LE intensifying screens (Kodak Scientific Imaging System, Cambridge) and individual metabolites quantified using a Bio-Rad GS-525 Phosphor-imager which had been calibrated with known amounts of ^{14}C metabolites. The identity of metabolites was confirmed by their co-chromatography in both TLC systems with authentic unlabelled or ^{14}C-labelled standards. In addition, radioactive metabolites were co-chromatographed with authentic standards using a reverse-phase HPLC column (Symmetry C18, 3.5 μm, 4.6×30 mm). The initial solvent system of acetonitrile/1% (v/v) phosphoric acid (5:95; v/v) was changed after 2 ml to give a linear gradient to 100% acetonitrile at 7.6 ml. The flow rate was 0.8 ml.min^{-1}.

4.6. Enzyme extraction and assay

Frozen soybean tissue was ground to a fine powder using a pestle and mortar and suspended in 1.5 vol. homogenising buffer [200 mM Tris–HCl, pH 7.5, 2 mM $MgCl_2$, 5% (w/v) PVPP, 5 mM DTT, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 mM PMSF]. The slurry was strained through four layers of muslin and the filtrate centrifuged (20,000*g*, 10 min at 4 °C). The resulting supernatant was employed for enzyme assays.

For *Arabidopsis*, the frozen tissue was ground under liquid nitrogen using a pestle and mortar and the resulting powder was resuspended in 3 vol. extraction buffer [0.2 M Tris–HCl pH 8, 5% (w/v) PVPP, 1 mM DTT]. The preparation was filtered and centrifuged as above and proteins in the supernatant precipitated after adjusting to 70% (w/v) with respect to ammonium sulphate and collected by centrifugation (10,000*g*, 40 min). Protein pellets were resuspended in extraction buffer and desalted on a G25 column. In all cases, the protein concentration was determined using Coomassie dye Reagent (BioRad) and γ-globulin as reference protein.

4.7. Enzyme assays

For DCA-*N*-MT assays, the mixture consisted of 100 mM Bis-Tris Propane–HCl, pH 6.5, 300 μM DCA, 9 μM [2-^{14}C]-malonyl-CoA (3.7 kBq), 22 μM cold malonyl-CoA, 0.1% (w/v) BSA and 20 μg crude protein, in a final vol. of 100 μl. After incubation at 35 °C for 5 min, the reaction was stopped with of 3 μl glacial acetic acid and the malonylated conjugates partitioned into 200 μl ethyl acetate and 100 μl of the organic phase quantified by LSC.

The DCA-*N*-GT assay mixture consisted of 200 mM Tris–HCl, pH 8.0, 5 μl of 1 mM DCA, 10 μl of [UL-^{14}C]-UDP-glucose (962 Bq) and 100 μg protein, in a final vol. of 75 μl. After incubation at 30 °C for 20 min, 125 μl Tris–HCl, pH 8.0 was added and the glucosylated conjugates were partitioned into 200 μl ethyl acetate and 100 μl quantified by LSC. Controls consisted of incubating the protein extract under identical conditions except that DCA was substituted with methanol.

To prepare radioactive reference metabolites of M-DCA and G-DCA, the respective organic phases from the reactions were pooled and then concentrated under a stream of N_2 prior to analysis by TLC.

Acknowledgements

We gratefully acknowledge Aventis CropScience UK and BBSRC for CASE PhD studentships awarded to S-h L, CL and MB. Rothamsted Research receives grant-aided support from the BBSRC of the UK.

References

Bartha, R., You, I.-S., Saxena, A., 1983. Humus-bound residues of phenylamide herbicides: their nature, persistence and monitoring, Vol. 3. In: Miyamoto, J., Kearney, P.C. (Eds.), Pesticide Chemistry. Pergamon, Oxford, pp. 345–350.

Barz, W., Mackenbrock, U., 1994. Constitutive and elicitation induced metabolism of isoflavones and pterocarpans in chickpea (*Cicer arietinum*) cell suspension cultures. Plant Cell Tiss. Org. Cult. 38, 199–211.

Bockers, M., Rivero, C., Thiede, B., Jankowski, J., Schmidt, B., 1994. Uptake, translocation, and metabolism of 3,4-dichloroaniline in soybean and wheat plants. Z. Naturforsch. 49c, 719–726.

Brazier, M., Cole, D.J., Edwards, R., 2002. *O*-Glucosyltransferase activities toward phenolic natural products and xenobiotics in wheat and herbicide-resistant and herbicide-susceptible black-grass (*Alopecurus myosuroides*). Phytochemistry 59, 149–156.

Frear, D.S., 1968. Herbicide metabolism in plants. I. Purification and properties of UDP-glucoside: arylamine *N*-glucosyl-transferase from soybean. Phytochemistry 7, 381–390.

Gareis, C., Rivero, C., Schuphan, I., Schmidt, B., 1992. Plant metabolism of xenobiotics. Comparison of the metabolism of 3,4-dichloroaniline in soybean excised leaves and soybean cell suspension cultures. Z. Naturforsch. 47c, 829–832.

Harborne, J.B., Self, R., 1987. Malonylated cyanidin 3-glucosides in *Zea mays* and other grasses. Phytochemistry 26, 2417–2418.

Harms, H., Langbartels, C., 1986. Standardised plant cell suspension test systems for an ecotoxicologic evaluation of the metabolic fate of xenobiotics. Plant Sci. 45, 157–165.

Harvey, P.J., Campanella, B.F., Castro, P.M.L., Harms, H., Lichtfouse, E., Schäffner, A.R., Smrcek, S., Werck-Reichhart, D., 2002. Phytoremediation of polyaromatic hydrocarbons, anilines and phenols. Environ. Sci. Pollut. Res. 9, 29–47.

Hinder, B., Schellenberg, M., Rodoni, S., Ginsburg, S., Vogt, E., Martinoia, E., Matile, P., Hörtensteiner, S., 1996. How plants dispose of chlorophyll catabolites. Directly energized uptake of tetrapyrrolic breakdown products into isolated vacuoles. J. Biol. Chem. 271, 27233–27236.

Höhl, H.-U., Barz, W., 1995. Metabolism of the insecticide Phoxim in plants and suspension cultures of soybean. J. Agric. Food Chem. 43, 1052–1056.

Köster, J., Bussmann, R., Barz, W., 1984. Malonyl-Coenzyme A: isoflavone 7-*O*-glucoside-6″-*O*-malonyltransferase from roots of chick pea (*Cicer arietinum* L.). Arch. Bichem. Biophys. 234, 513–521.

Lu, Y.P., Li, Z.S., Drozdowicz, Y.M., Hörtensteiner, S., Martinoia, E., Rea, P.A., 1998. AtMRP2, an Arabidopsis ATP binding cassette transporter able to transport glutathione S-conjugates and chlorophyll catabolites: functional comparisons with AtMRP1. Plant Cell. 10, 267–282.

Mackenbrock, U., Vogelsang, R., Barz, W., 1992. Isoflavone and pterocarpan malonylglucosides and β-1,3-glucan- and chitin-hydrolases are vacuolar constituents in chickpea (*Cicer arietinum* L.). Z. Naturforsch. 47c, 815–822.

Mackenbrock, U., Gunia, W., Barz, W., 1993. Accumulation and metabolism of medicarpin and maakiain malonylglucosides in elicited chickpea (*Cicer arietinum* L.) cell suspension cultures. J. Plant Physiol. 142, 385–391.

Marrs, K.A., 1996. The functions and regulation of glutathione *S*-transferases in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 47, 127–158.

Matern, U., 1983. Acylhydrolases from parsley (*Petroselinum hortense*). Relative distribution and properties of four esterases hydrolyzing malonic acid hemiesters of flavonoid glucosides. Arch. Biochem. Biophys. 224, 261–271.

Matern, U., Heller, W., Himmelspach, K., 1983. Conformational changes of apigenin 7-*O*-(6-*O*-malonylglucoside), a vacuolar pigment from parsley, with solvent composition and proton concentration. Eur. J. Biochem. 133, 439–448.

Matern, U., Feser, C., Heller, W., 1984. *N*-malonyltransferases from peanut. Arch. Biochem. Biophys. 235, 218–227.

Parris, G.E., 1980. Environmental and metabolic transformations of primary aromatic amines and related compounds. Residue Rev. 76, 1–30.

Robineau, T., Batard, Y., Nedelkina, S., Cabello-Hurtado, F., LeRet, M., Sorokine, O., Didierjean, L., Werck-Reichhart, D., 1998. The chemically inducible plant cytochrome P450 CYP76B1 actively metabolizes phenylureas and other xenobiotics. Plant Physiol. 118, 1049–1056.

Sandermann, H., Scheel, D., v.d. Trenck, T., 1983. Metabolism of environmental chemicals by plants—copolymerization into lignin. Appl. Polym. Sci.: Appl. Polym. Symp. 37, 407–420.

Sandermann, H., 1994. Higher plant metabolism of xenobiotics: the "green liver" concept. Pharmacogenetics 4, 225–241.

Sandermann, H., Schmitt, R., Eckey, H., Bauknecht, T., 1991. Plant biochemistry of xenobiotics: isolation and properties of soybean *O*- and *N*-glucosyl and *O*- and *N*-malonyltransferases for chlorinated phenols and anilines. Arch. Biochem. Biophys. 287, 341–350.

Schmidt, B., Rivero, C., Thiede, B., 1995. 3,4-dichloroaniline *N*-glucosyl and *N*-malonyltransferase activities in cell cultures and plants of soybean and wheat. Phytochemistry 39, 81–94.

Schmidt, B., Thiede, B., Rivero, C., 1994. Metabolism of the pesticide metabolites 4-nitrophenol and 3,4-dichloroaniline in carrot (*Daucus carota*) cell suspension cultures. Pest. Sci. 40, 231–238.

Schmitt, R., Kaul, J., Trenck, T.V.D., Schaller, E., Sandermann, H., 1985. β-D-glucosyl and *O*-malonyl-β-D-glucosyl conjugates of pentachlorophenol in soybean and wheat: identification and enzymatic synthesis. Pest. Biochem. Physiol. 24, 77–85.

Still, G.G., 1968. Metabolism of 3,4-dichloroporionanilide in plants: the metabolic fate of the 3,4-dichloroaniline moiety. Science 159, 992–993.

Tommasini, R., Vogt, E., Schmid, J., Fromentau, M., Amrhein, N., Martinoia, E., 1997. Differential expression of genes coding for ABC transporters after treatment of *Arabidopsis thaliana* with xenobiotics. FEBS Lett. 411, 206–210.

Tommasini, R., Vogt, E., Fromenteau, M., Hörtensteiner, S., Matile, P., Amrhein, N., Martinoia, E., 1998. An ABC-transporter of *Arabidopsis thaliana* has both glutathione-conjugate and chlorophyll catabolite transport activity. Plant J. 13, 773–780.

Van der Trenck, K.T., Hunkler, D., Sandermann, H., 1981. Incorporation of chlorinated anilines into lignin. Z. Naturforsch. C 36, 714–720.

Winkler, R., Sandermann, H., 1989. Plant metabolism of chlorinated anilines: isolation and identification of *N*-glucosyl and *N*-malonyl conjugates. Pest. Biochem. Physiol. 33, 239–248.

Winkler, R., Sandermann, H., 1992. *N*-glucosyl conjugates of chlorinated anilines—spontaneous formation and cleavage. J. Agric. Food Chem. 40, 2008–2012.

Yang, S.F., Hoffman, N.E., 1984. Ethylene biosynthesis and its regulation in higher plants. Annu. Rev. Plant Physiol. 35, 155–189.

APPENDIX 2

Mascot search results of differentially expressed spots after DiGE analysis